Oxidative Medicine and Cellular Longevity

Molecular Mechanisms of Oxidative and Nitrosative Stress Regulation in Psychiatric Disorders

Lead Guest Editor: Magdalena Kotańska
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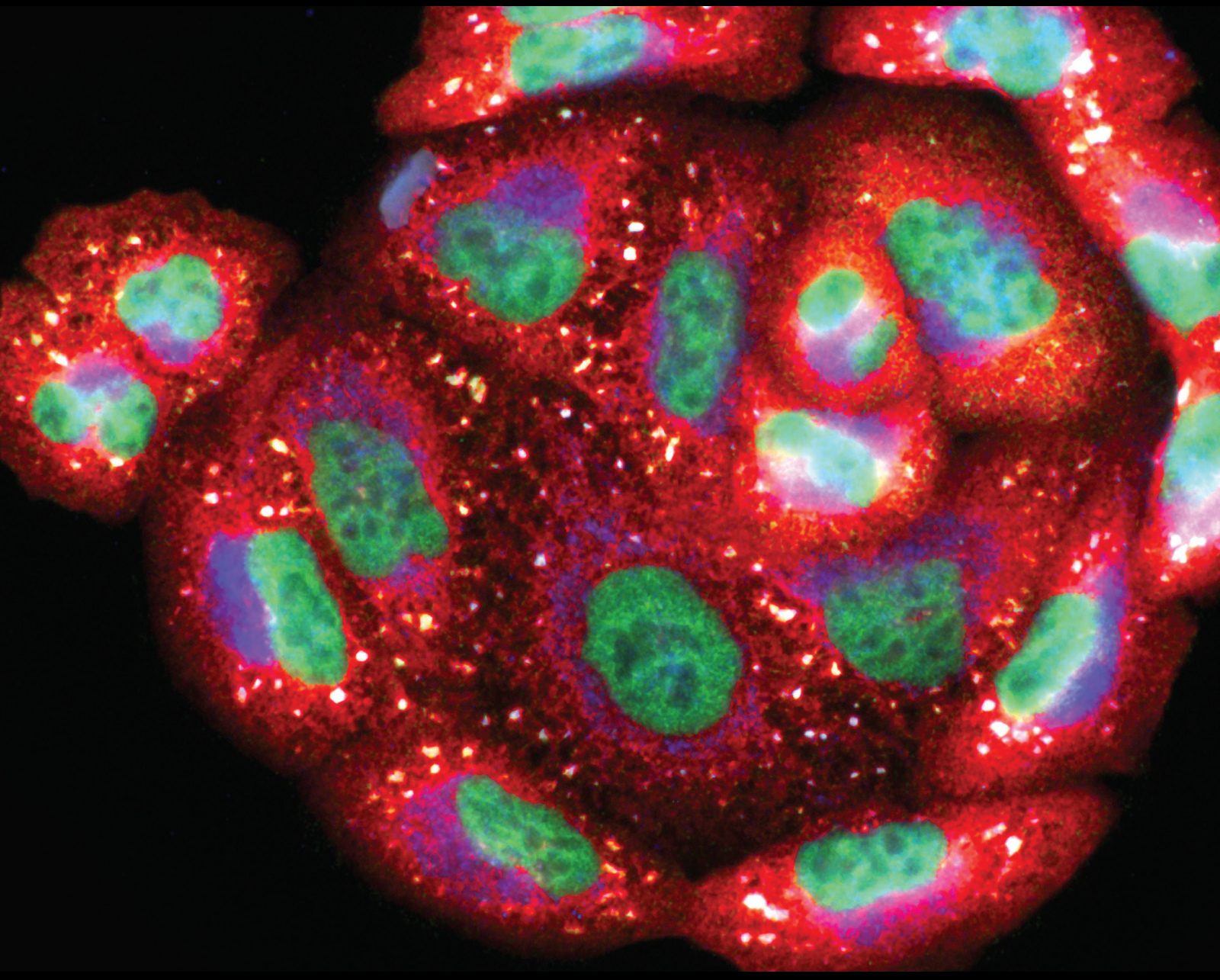

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Retraction

Retracted: Carveol Promotes Nrf2 Contribution in Depressive Disorders through an Anti-inflammatory Mechanism

Oxidative Medicine and Cellular Longevity

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Peer-review manipulation

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] A. J. Muhammad, L. Hao, L. T. Al Kury et al., "Carveol Promotes Nrf2 Contribution in Depressive Disorders through an Anti-inflammatory Mechanism," *Oxidative Medicine and Cellular Longevity*, vol. 2022, Article ID 4509204, 16 pages, 2022.

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Research Article

Hydroalcoholic Leaf Extract of *Isatis tinctoria* L. *via* Antioxidative and Anti-Inflammatory Effects Reduces Stress-Induced Behavioral and Cellular Disorders in Mice

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Stress that can occur at different levels of a person's life can cause and exacerbate various diseases. Oxidative stress and inflammation underlie this process at the cellular level. There is an urgent need to identify new and more effective therapeutic targets for the treatment of stress-induced behavioral disorders and specific drugs that affect these targets. *Isatis tinctoria* L. is a herbaceous species in the Brassicaceae family. Due to its potential antioxidant, nitric oxide- (NO-) inhibiting, anti-inflammatory, and neuroprotective properties, *I. tinctoria* could be used to treat depression, anxiety, and stress resistance. Hence, the present study is aimed at delineating whether administration of *I. tinctoria* leaf extract may improve stress-induced disorders in mice. A set of four behavioral tests was selected that together are suitable for phenotyping acute restraint stress-associated behaviors in mice, namely locomotor activity, social integration, dark/light box, and splash tests. The plasma and brains were collected. A brain-derived neurotrophic factor, tumor necrosis factor-alpha, C-reactive protein, corticosterone, NO, reactive oxygen species levels, superoxide dismutase and catalase activity, and ferric-reducing antioxidant power were measured. In mice stressed by immobilization, decreased locomotor activity, anxiety-like behavior, and contact with other individuals were observed, as well as increased oxidative stress and increased levels of nitric oxide in the brain and plasma C-reactive protein. A single administration of *I. tinctoria* leaf extract was able to reverse the behavioral response to restraint by a mechanism partially dependent on the modulation of oxidative stress, neuroinflammation, and NO reduction. In conclusion, *Isatis tinctoria* hydroalcoholic leaf extract can reduce stress-induced behavioral disturbances by regulating neurooxidative, neuronitrosative, and neuroimmune pathways. Therefore, it could be recommended for further research on clinical efficacy in depression and anxiety disorder treatment.

1. Introduction

Depression, anxiety, and stress-related disorders are the most prevalent psychiatric disorders, accounting for a disproportionate share of mental illnesses in highly developed countries [1]. Stress levels throughout a person's life may contribute to the development of a variety of diseases, both central and peripheral (hypertension, ischemic heart disease, muscle spasms with subsequent chronic pain, cancer, inflammation, and weight disorders), and are often fatal in many people [2–6]. Anxiety and depression have raised to prominence as major health problems on a global scale in recent years. As a result, there is an urgent need to identify new and more effective therapeutic goals for the treatment of stress-induced behavioral disorders, as well as specific drugs that affect these goals. It is critical not only from a medical but also from a socioeconomic standpoint.

Newer findings suggest the crucial role that the immune system, oxidative, and nitrative stress play in the development of psychiatric disorders such as anxiety and major depression [7].

It is well known that brain tissue is particularly susceptible to oxidative damage, compared to other organs, due to the relatively high content of iron and peroxide fatty acids, in addition to its limited antioxidative capacity [8]. Reactive oxygen species are produced during physiological processes, but when present in excess or under conditions of decreased availability of antioxidant defenses, they can cause structural and functional changes that produce cell injury [9].

NO is a messenger molecule that is widely distributed in cells and can influence a wide variety of physiological and pathological processes. Overproduction of NO causes tissue damage and can cause various inflammatory diseases. However, NO also plays an important neuromodulatory role in the central nervous system. It is synthesized from L-arginine by nitric oxide synthase and is involved in neurotransmission, synaptic plasticity, learning, pain sensation, aggression, stress response, and depression [10]. Some evidence has shown that lowering nitric oxide levels in the hippocampus can induce antidepressant-like effects by increasing stress-coping activity, thereby confirming that endogenous hippocampal nitric oxide plays a role in the neurobiology of stress and depression [7, 11]. Literature data indicate that nitric oxide synthase inhibitors increased extracellular serotonin and dopamine levels in the rat ventral hippocampus, while L-arginine acted the opposite, thereby demonstrating the role of endogenous nitric oxide in regulating serotonin and dopamine levels in the hippocampus [12].

One of the most recent and important findings is that the elements of the immune system that mediate inflammation may be closely related to behavioral disorders induced by stress. Inflammation is usually viewed as the body's primary response to physical damage or infection. However, there is now substantial evidence that mental stress can cause a significant increase in inflammation. In turn, worsening inflammation can trigger profound behavioral changes that include the initiation of depressive symptoms such as sad mood, anhedonia, fatigue, psychomotor retardation, and sociobehavioral withdrawal [13].

Different nutraceuticals, including flavonoids, show antidepressant activities and may reduce stress-induced behaviors in animal models and humans as well [7, 14, 15].

Isatis tinctoria L. (woad) is an herbaceous species in the Brassicaceae family that is widely distributed throughout the world and has been valued for centuries for its dyeing properties. The root and leaves have a long and well-documented history of use as a medicinal raw material in both Western and Eastern cultures [16, 17]. The *I. tinctoria* root also has monographs in both the European and Chinese pharmacopeias. Today, it is widely used in Traditional Chinese Medicine (TCM) to treat a variety of diseases, including hepatitis, influenza, and bacterial infections of the respiratory system [18].

The interest in this plant has rapidly grown due to its phytochemical profile, rich in bioactive compounds including glucosinolates, flavonoids, and indole, quinazolone, and quinoline alkaloids, which have anti-inflammatory, antitumor, antimicrobial, analgesic, and antioxidant properties [16]. Numerous phytochemical studies of the root and leaves of *I. tinctoria* indicate differences in their chemical composition. Depending on the extraction and separation methods, particular groups of metabolites are dominant [19, 20]. In in vitro and in vivo studies both the root and leaf extracts from *I. tinctoria* show anti-inflammatory properties by inhibiting proinflammatory cytokines [21, 22] and suppression of nitric oxide (NO) production, which at least in part explain its impact on inflammatory and oxidative pathways [23–25]. *Isatis tinctoria* antioxidants' effects against free radicals are further supported by many in vitro assays revealing its strong scavenging activity as well as its good reducing power [16]. More recent research has focused on its neuroprotective properties, for example, against hydrogen peroxide-induced cell injury (H_2O_2) [26].

Due to the high concentration of flavonoids and other phenolic compounds in *I. tinctoria* leaves, which have antioxidant, NO inhibitory, anti-inflammatory, and neuroprotective properties, the extract of this plant may be useful to reduce depressive and anxiety symptoms, as well as improve resilience to stress, potentially providing an alternative to conventional antidepressants. Nevertheless, to date, no studies have examined the effects of treatment with *I. tinctoria* on depressive-like behaviors. Therefore, this study was carried out to delineate the effects of *I. tinctoria* on stress-induced behavioral disorders in mice.

2. Materials and Methods

2.1. Plant material. Cauline leaves were picked from *Isatis tinctoria* L. growing wild around Acireale (Catania, Sicily, Italy). The taxonomic identification of the plant materials was confirmed by Prof. Salvatore Ragusa; specimens are deposited in the Herbarium of the Department of Scienze della Salute, University "Magna Graecia" of Catanzaro (Italy), under accession number no. 327/11. The dried plant material was extracted in accordance with the procedure reported by Taviano et al. [27]. Finally, the hydroalcoholic extract (MeOH 70%) was evaporated to dryness *in vacuo*, and the yield, referred to as 100 g of dried plant material,

was 24.32%. The phytochemical analysis of the extract was previously performed by HPLC-PDA-ESI-MS [27].

2.2. Extract and Chemicals. Three different concentrations of *I. tinctoria* leaf extract (IT) were tested *in vivo*. To prepare the solution for injecting mice, dried extracts of *I. tinctoria* leaves were suspended in 1% Tween 80 solution (Sigma-Aldrich, France) to obtain the final concentration. The solutions were freshly prepared before the experiment and administered in a single dose to mice intraperitoneally (i.p.) at the following doses: 50 mg/kg, 100 mg/kg, or 500 mg/kg b.w. Vehicle (1% Tween 80) was administered i.p. at a volume of 10 mL/kg. Bupropion at a dose of 10 mg/kg b.w. was chosen as the reference compound [47]. Bupropion inhibits dopamine reuptake and increases its availability in the synaptic cleft. Dopamine is associated with motivation, reward, and hedonic states, and therefore, increasing dopamine levels could improve depressive-like symptoms [28]. In our study, we chose bupropion because it is suggested to exert antidepressant effects and it is expected to reduce oxidative stress [29] and inflammation [30]. This drug was used in the treatment of posttraumatic stress disorder [31].

Heparin was purchased from Polfa Warszawa S.A. (Warsaw, Poland).

2.3. Animals. Six-week-old, male Albino Swiss mice, CD-1, weighing 20–22 g, were used. The animals (160 subjects) were obtained from the Animal House of the Pharmaceutical Faculty of Jagiellonian University and kept in environmentally controlled rooms, in standard cages lit by artificial light for 12 hours each day. Animals were free to access food and water, except for the time of the acute experiment. The randomly established experimental groups consisted of 8 mice. All animal care and experimental procedures were carried out in accordance with European Union and Polish legislation acts concerning animal experimentation and were approved by the Local Ethics Committee at the Jagiellonian University in Cracow, Poland (No.: 473/2020, 511/2021, 545B/2021).

2.4. Behavioral Studies. Behavioral studies were carried out in a quiet and stress-free environment, and the animals were handled several days before the actual study to acquaint them with the experimenter. The temperature and lighting conditions of the facility were kept constant. Mice are more active during the dark phase of their light-dark cycle, and so, the animals were tested at approximately the same time of day for better within-study and cross-study comparisons.

All scheduled tests are mild on animals (light, pain-free). Therefore, it was planned to carry out the tests in pairs in one mouse to save the number of animals in accordance with the recommendations of the Ethics Committee for Animal Research. Thus, spontaneous activity was assessed 5 minutes before the social integration test, whereas the dark/light box test was run 5 minutes before the splash test. The experiments schemes are shown in Figures 1 and 2.

2.4.1. Locomotor Activity Test. Locomotor activity was individually recorded for each animal using specifically designed activity cages made of clear Perspex (40 cm × 40 cm × 31 cm, Activity Cage 7441, Ugo Basile, Italy) [32]. The cages came supplied with I.R. horizontal beam emitters connected to a counter which records the light-beam interruptions. The prepared suspensions were administered as i.p. injections 30 min before testing. The control animals received i.p. injections of vehicles. Each mouse was placed in a cage for a 30-minute habituation period. After that time, the number of breaks in the photobeams was measured for 5 minutes which is the same time as the observation period used in other tests.

2.4.2. Social Integration Test. The social integration test was carried out according to a procedure described by Kraeuter and colleagues with minor modifications [33].

The test mouse was placed into the center zone of the open-field box (light-grey colored 42 × 42 × 42 cm polyvinyl chloride (PVC) chamber with a marked 20.5 × 20.5 central zone), immediately before placing the stimulus mouse in the same area of the box. Mice behaviors were recorded by the camera for 5 minutes. The number of approaches of the test mouse versus stimulus mouse was counted.

2.4.3. Dark/Light Box Test. The dark/light box test was carried out according to a procedure described by Bourin and Hascoet and Kulesskaya and Voikar with minor modifications [34, 35]. The test was carried out in the open-field arena with a white floor (40 cm × 40 cm × 31 cm, Activity Cage 7441, Ugo Basile, Italy) equipped with infrared light sensors (at 1.5 cm intervals) detecting horizontal and vertical activity. The dark insert (with black walls and lid, nontransparent for visible light) was used to divide the arena into two parts of size: 40 × 13 cm (dark box) and 40 × 27 cm (light box). An opening (width 3.5 cm and height 5 cm) in the wall of the insert allows animals' free movement from one compartment to another. The light side was illuminated (illumination in the center of the light compartment ~1000 lx). The animal was released in the center of the light compartment (facing away from the opening) and allowed to explore the arena for 5 min. The distance traveled in light, the number of entrances to the dark box, and the time spent in different compartments were recorded.

2.4.4. Splash Test. The splash test was carried out in a standard mouse cage according to a procedure described by Casaril and colleagues [36].

Briefly, animals were splashed on the dorsal coat with 10% sucrose solution to induce grooming behavior, defined as cleaning of the fur by licking, scratching, or head washing. The latency of the first grooming and the total grooming time were measured for 5 min immediately after the splash of sucrose solution and scored by trained personnel blinded to the treatments.

2.4.5. Acute Restraint Stress (ARS) Model. The physical restraint was performed in mice as previously reported by Casaril and colleagues [36].

Briefly, mice were subjected to immobilization for 240 min using an individual rodent restraint device made of Plexiglas, restraining all physical movement and causing

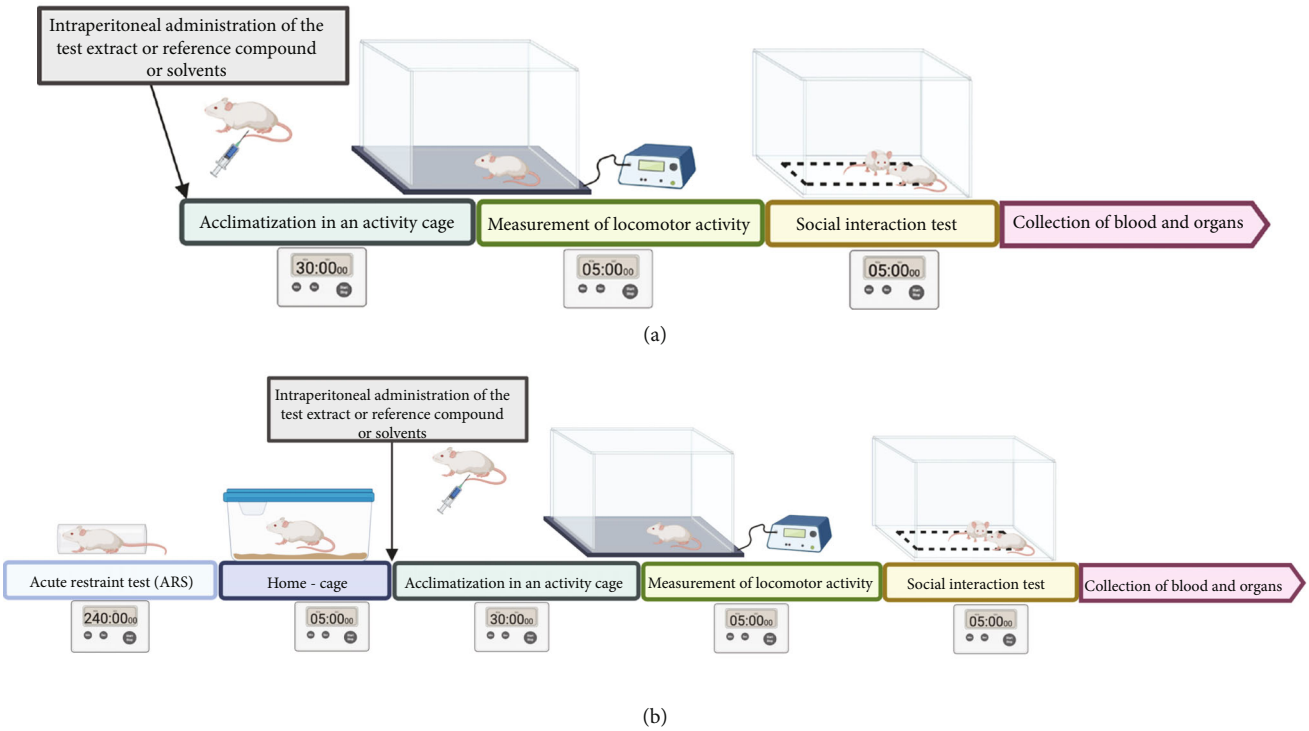

Figure 1: Scheme of locomotor activity test and social integration test (a) without the ARS model and (b) after the ARS model.

no pain. Mice were deprived of food and water during physical stress. After restraint stress, the mice were put back in their home cage, and 5 minutes later, they received tested extracts, vehicle, or reference compound. They were subjected to behavioral tests 30 minutes later.

2.5. Collecting Plasma and Brains. At the end of the experiment, 60 min after administration IT, vehicle, or bupropion and 20 min after i.p. administration of heparin (2500 units/mice), the blood was collected after decapitation and then centrifuged at 600 × g (15 min, 4°C) to obtain plasma. The brain (only the cerebral cortex with the hippocampus) was collected on ice immediately after decapitation and freezing in liquid nitrogen and then placed in a freezer (-80°C) until biochemical assays are performed. On the day of the assays, brain parts were weighed, and homogenates were prepared by homogenization at the ratio of 1 g of the tissue in 4 ml of 0.1 M phosphate buffer, pH 7.4 using an IKA-ULTRA-TURRAX T8 homogenizer.

2.6. Biochemical Analysis. For the determination of brain-derived neurotrophic factor (BDNF), tumor necrosis factor-alpha (TNF-α), or C-reactive protein (CRP) levels, standard ELISA tests with spectrophotometric reading (Shanghai Sunred Biological Technology Co., Ltd, China) were applied. For the determination of superoxide dismutase (SOD) and catalase (CAT) activities and corticosterone or nitric oxide (NO) levels, standard enzymatic and spectrophotometric tests were used (CaymanChem, USA).

2.6.1. Reactive Oxygen Species (ROS). ROS were assayed according to the method of Bondy and Guo [37], using DCFH-DA which is deesterified in brain homogenates to 2′,7′-dichlorofluorescein acid and then oxidized by ROS to fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, to 10 μl of homogenate, 990 μl of 0.1 M phosphate buffer (pH 7.4) and 10 μl of 1.25 M DCFH-DA dissolved in ethanol were added. The reaction mixture was incubated at 37°C for 30 min, protecting the samples from light. The measurements were conducted using a fluorometer at wavelengths: A_{ex} = 488 nm and A_{em} = 525 nm. ROS were evaluated using a standard curve for 1 μM DCF.

2.6.2. Ferric-Reducing Antioxidant Power (FRAP) assay. The assay was performed according to Benzie and Strain [38] with some modifications. The FRAP working solution was prepared before the start of the analysis: 0.3 mol acetate buffer (pH 3.6), 0.01 mol TPTZ (2,4,6-tripyridyl-s-triazine; Sigma-Aldrich) in 0.04 mol HCl (POCh, Poland), and 0.02 M $FeCl_3 \times 6H_2O$ in water (iron (III) chloride hexahydrate; Chempur, Poland) were mixed in a volumetric ratio of 10 : 1 : 1 and protected from light.

Next, 20 μl of the plasma sample tested, brain homogenate, or $FeSO_4 \times 7H_2O$ solution was mixed with 180 μl of the FRAP working solution. The mixtures obtained were incubated at 37°C for 30 minutes, and their absorbance was measured at 593 nm. $FeSO_4 \times 7H_2O$ (100–1000 μM/l) was used for a calibration curve. Deionized water with FRAP solution was used as a blank.

2.7. Statistical Analysis. Statistical calculations were performed using GraphPad Prism 6 software (GraphPad Software, USA). The results are expressed as mean + $\Delta/2$, where Δ is a width of the 95% confidence interval (CI), $n = 6 - 8$.

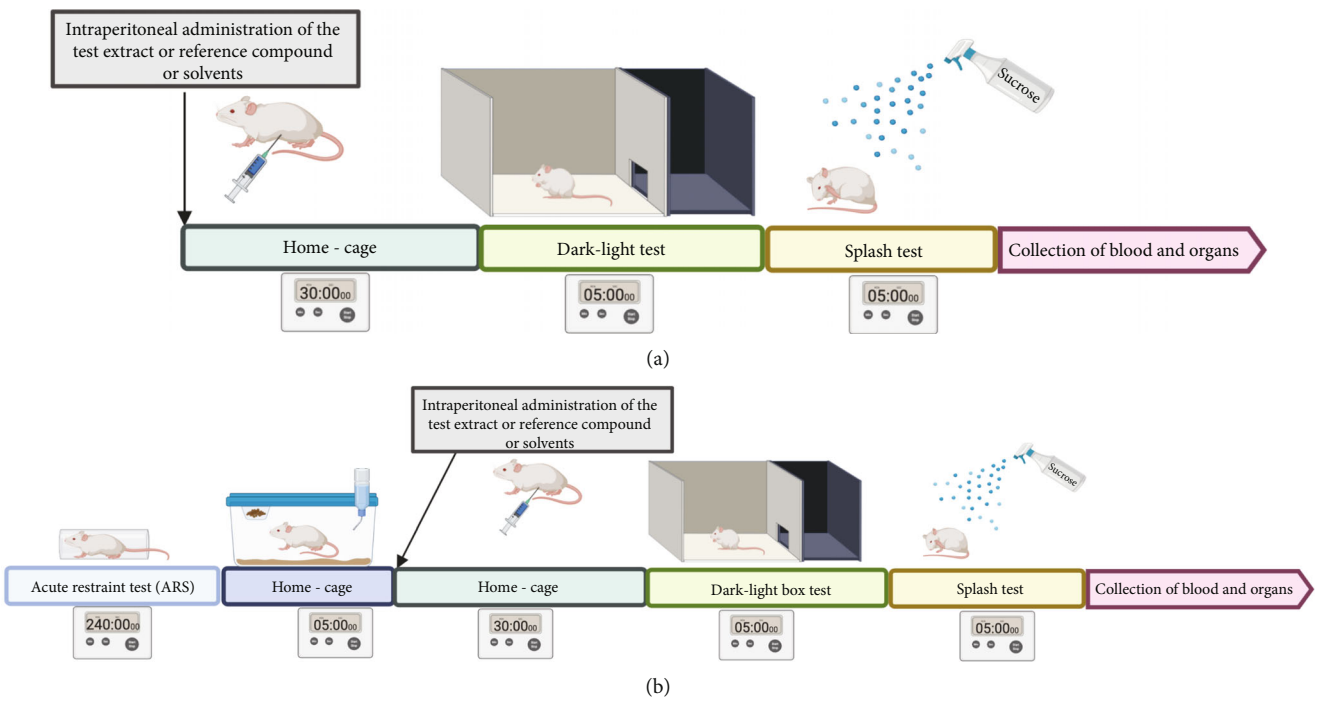

Figure 2: Scheme of dark/light box test and splash test (a) without the ARS model and (b) after the ARS model.

Statistical significance was calculated using one-way ANOVA, Dunnett's, or Tukey post hoc test (respectively, when there are one or two control groups). Differences were considered statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

3. Results

3.1. Influence of IT on Locomotor Activity. IT administered at a dose of 50 mg/kg body weight (b.w.) significantly ($p < 0.05$) decreased the locomotor activity, compared to the activity determined in the control group. The remaining doses 500 mg/kg b.w. and 100 mg/kg b.w. did not cause a significant effect on the locomotor activity. The reference compound bupropion, administered at a dose of 10 mg/kg b.w., significantly ($p < 0.05$) increased the locomotor activity. The results are shown in Figure 3(a).

In the acute restraint stress (ARS) model, in stressed control mice, locomotor activity was significantly ($p < 0.05$) lower than in the naïve control group. However, this significant decrease in activity was not observed in all groups administered IT or the reference compound. In addition, in the group administered with the highest dose (500 mg/kg b.w.), locomotor activity was significantly augmented ($p < 0.05$) compared to the stressed control group. The results are shown in Figure 3(b).

3.2. Influence of IT on Incidents of Social Interaction. In naïve mice, all doses of IT tested (500 mg/kg b.w., 100 mg/kg b.w., and 50 mg/kg b.w.) as well as bupropion did not significantly affect the number of approaches of the test mice versus stimulus mice. The results are shown in Figure 4(a).

In the ARS model, the number of approaches initiated by the mice in the stressed control group was significantly lower ($p < 0.05$) compared to the naïve control group. For all tested doses of IT and bupropion, an increase in the number of approaches initiated by the test mice towards the stimulus mice was observed compared to the stressed control group (no significant differences compared to the nonstress control group). However, only bupropion increased the number of contacts initiated at a significant level ($p < 0.05$). The results are shown in Figure 4(b).

3.3. Influence of IT on Behaviors in an Unknown Space (Dark/Light Box Test). In naïve mice, all the IT-treated and bupropion-treated groups showed no significant changes in the number of passes through the hole between light and dark fields. The results are shown in Figure 5(a).

The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's or Tukey's post hoc test was used to calculate the significance of differences between the groups, $n = 7 - 8$. * Significant difference vs. the control group without stress. $^{\wedge}$Significant difference vs. the control group with stress. Significance level: $^{*,\wedge}p < 0.05$; $\wedge\wedge p < 0.01$; $^{***,\wedge\wedge\wedge}p < 0.001$.

In the ARS model, the number of transitions between light and dark box was significantly lower ($p < 0.001$) in the stressed control mice than in the naïve control group. IT at a dose of 100 mg/kg b.w. had no significant effect on the number of passes; the number of counts in this group was comparable to the number of counts recorded in the stressed control group. After administration of a dose of 50 mg/kg b.w. or bupropion, there were significantly fewer passes compared to the number of passes observed in the naïve control group. In addition, after administration of IT in doses of 500 mg/kg b.w. and 50 mg/kg b.w. or bupropion,

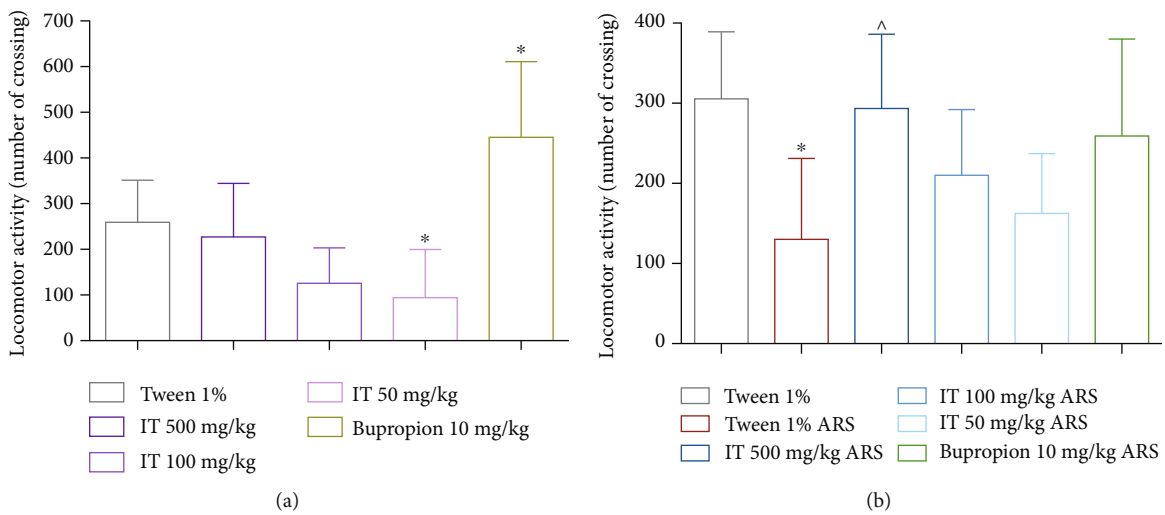

Figure 3: The effect of *Isatis tinctoria* leaf extract on locomotor activity in mice: (a) without stress (naïve mice), (b) after stress. ARS: acute stress model, IT: *Isatis tinctoria* leaf extract. The results are presented as the mean of the measurements ± $\Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA was used to calculate the significance of differences between groups, followed by Dunnett's or Tukey's post hoc test, $n = 7 - 8$. *Significant difference vs. the control group without stress. ∧Significant difference vs. the control group with stress. significance level: *,∧$p < 0.05$.

the nember of light-field and dark-field passes significantly increased compared to the number of passes in stressed control mice ($p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively). The results are shown in Figure 5(b).

IT administered to mice at the following doses, 500 mg/kg b.w., 100 mg/kg b.w., 50 mg/kg b.w., or bupropion, did not significantly affect the locomotor activity of mice in light box, both in naïve and stressed control mice. The results are shown in Figures 5(c) and 5(d).

In naïve mice, in all IT-treated groups, no significant influence of the tested extracts was observed regarding the time spent by the mice in the dark field of the activity cage. The reference compound, bupropion, also did not significantly affect the time spent by mice in the dark. The results are shown in Figure 5(e).

In the ARS model, in the stressed control mice, the time spent in the dark field of the cage during one stay was statistically significantly longer ($p < 0.001$) than in the naïve control group. In all groups treated with IT or bupropion, the time spent in the dark field of the cage during one stay was shorter compared to the stressed control group (no significant differences compared to the control group without stress control group), whereas only at a dose of 500 mg/kg the reduction in time was significant, $p < 0.001$. The results are shown in Figure 5(f).

3.4. Influence of IT on Behaviors during Splash Test. In naïve mice, IT administered at the highest dose (500 mg/kg b.w.) significantly ($p < 0.05$) delayed the start of grooming behavior compared to the time measured for mice in the control group. The reference compound, bupropion, also significantly ($p < 0.05$) prolonged the time to start grooming in mice. The remaining IT doses, 100 mg/kg b.w. and 50 mg/kg b.w., did not significantly extend this time. The results are shown in Figure 6(a).

In stressed mice, no elongation of the interval between the splash of sucrose and the first grooming behavior was detected compared to the naïve control mice. All doses of IT or bupropion did not affect the time to start grooming compared to the stressed control group. IT at doses 500 mg/kg b.w. and 100 mg/kg b.w. significantly ($p < 0.05$) prolonged the time to the first grooming behavior compared to naïve control mice. IT dose 50 mg/kg b.w. or bupropion did not significantly extend this time. The results are shown in Figure 6(b).

In naïve mice, no significant effect of the tested extract was observed on the grooming time of the mice during the 5 min splashing test. The reference compound, bupropion, also did not significantly affect grooming time. The results are shown in Figure 6(c).

In the ARS model, in the stressed control mice, grooming time was significantly longer ($p < 0.05$) than in the naïve control group. In the groups with stress given IT at a dose of 50 mg/kg b.w., the grooming time of the mice compared to the naïve control group was also significantly longer ($p < 0.05$) and did not differ significantly from the level determined in the stressed control group. No significance was observed in the remaining groups given IT. Bupropion also did not exhibit a significant effect on this parameter. The results are shown in Figure 6(d).

In naïve mice, no significant influence of the tested extract on the number of washes was observed. The reference compound bupropion also did not significantly affect this number. The results are shown in Figure 6(e).

In the ARS model, the number of washes in the stressed control mice was significantly greater than in the naïve control group. After administration of IT at all doses tested (500 mg/kg b.w., 100 mg/kg b.w., and 50 mg/kg b.w.) as well as bupropion (10 mg/kg b.w.) to stressed mice, no significant change in the number of washes was observed

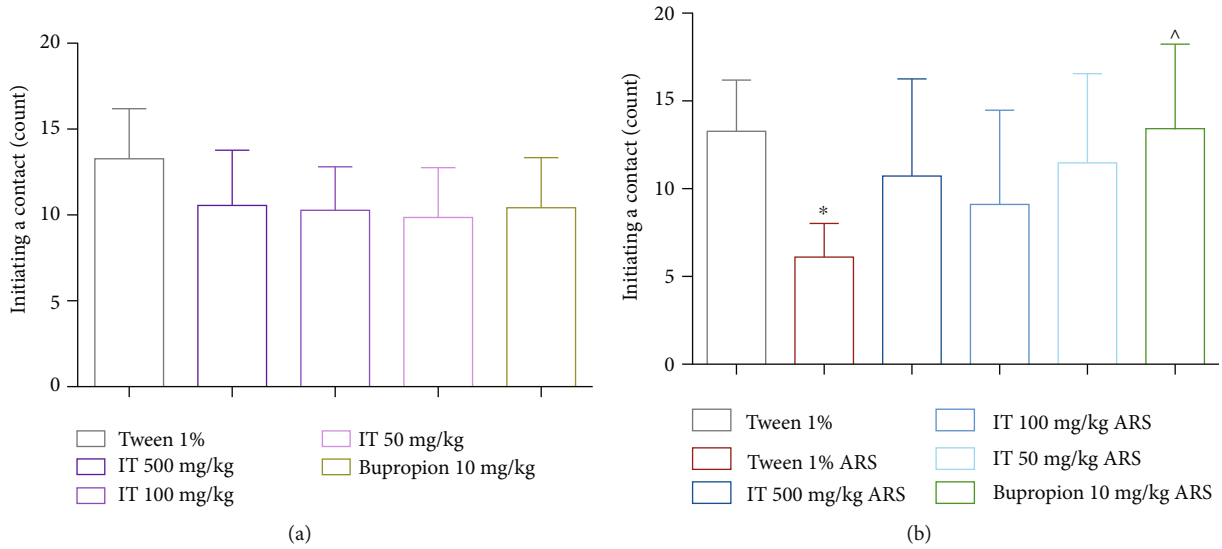

Figure 4: The effect of *Isatis tinctoria* leaf extract on the number of contacts initiated by test mice: (a) without stress (naïve mice), (b) after stress. ARS: acute stress model, IT: *Isatis tinctoria* leaf extract. The results are presented as the mean of the measurements ± $\Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's or Tukey's post hoc test was used to calculate the significance of differences between the groups, $n = 7 - 8$. *Significant difference vs. the control group without stress. ∧Significant difference vs. the control group with stress. Significance level: *,∧$p < 0.05$.

both compared to the stressed control group but also compared to the naïve control group. The results are shown in Figure 6(f).

3.5. Influence of IT on Plasma Corticosterone Level. The plasma level of corticosterone in the stressed control mice was significantly greater than in the naïve control group. In the group treated with the highest dose (500 mg/kg b.w.) of IT, the plasma corticosterone level significantly ($p < 0.05$) elevated in comparison to the level determined in the naïve control group. There were no significant differences in the corticosterone levels in the plasmas of the stressed mice treated with IT at doses 100 or 50 mg/kg b.w. compared to the levels of both control groups. The results are shown in Figure 7(a).

The results are presented as the mean of the measurements ± $\Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Tukey's post hoc test was used to calculate the significance of differences between the groups; $n = 7 - 8$. *Significant difference vs. control group without stress. ∧Significant difference vs. the control group with stress. Significance level: *$p < 0.05$; **,∧∧p < 0.01.

3.6. Influence of IT on Plasma or Brain BDNF Level. There were no significant differences in BDNF levels in the plasmas of the control groups. Significantly higher levels of plasma BDNF concentration were observed compared to plasma levels of both control groups only in the group treated with bupropion ($p < 0.01$). The results are shown in Figure 7(b). Significantly higher levels of BDNF were determined in the brain than in the brain of stressed mice only in the brain of mice treated with bupropion. The results are shown in Figure 7(c).

3.7. Influence of IT on Plasma CRP Level. In the plasma collected from mice in the stressed control group, a significantly ($p < 0.05$) higher level of CRP was determined compared to the level determined in the plasma collected from the mice naïve control group. After administration of 100 mg/kg of IT, and also of bupropion, the plasma level of CRP also increased significantly compared to the level measured in the control groups ($p < 0.001$). There were no significant differences in plasma CRP levels in the groups treated with IT at doses of 500 or 50 mg/kg b.w. The results are shown in Figure 8(a).

3.8. Influence of IT on Antioxidant Parameters. No significant differences in ferric-reducing ability were determined in the plasmas and brains collected from mice in this experiment. The results are shown in Figures 8(b) and 9(b).

The activity of SOD or CAT in the plasma collected from the stressed control animals resulted lower than the activity of these enzymes measured in the plasma collected from the naïve control group. The difference in SOD activity was significant ($p < 0.001$). After administration of the extract or bupropion, the activities of these enzymes determined in plasma did not differ significantly from the levels determined in the naïve control group (except CAT activity in the group treated with 500 mg/kg b.w.). Plasma activities of SOD from the extract- or bupropion-treated groups were significantly higher than the SOD activity in the plasma taken from the previously stressed control group ($p < 0.05$). CAT activity in the plasma collected from the IT-treated group at a dose of 500 mg/kg was significantly higher than the activity determined in the stressed control group ($p < 0.001$) and in the naïve control group ($p < 0.05$). The results are shown in Figures 8(c) and 8(d).

No significant differences in ROS level were determined in brains collected from mice in this experiment. The results are shown in Figure 9(a).

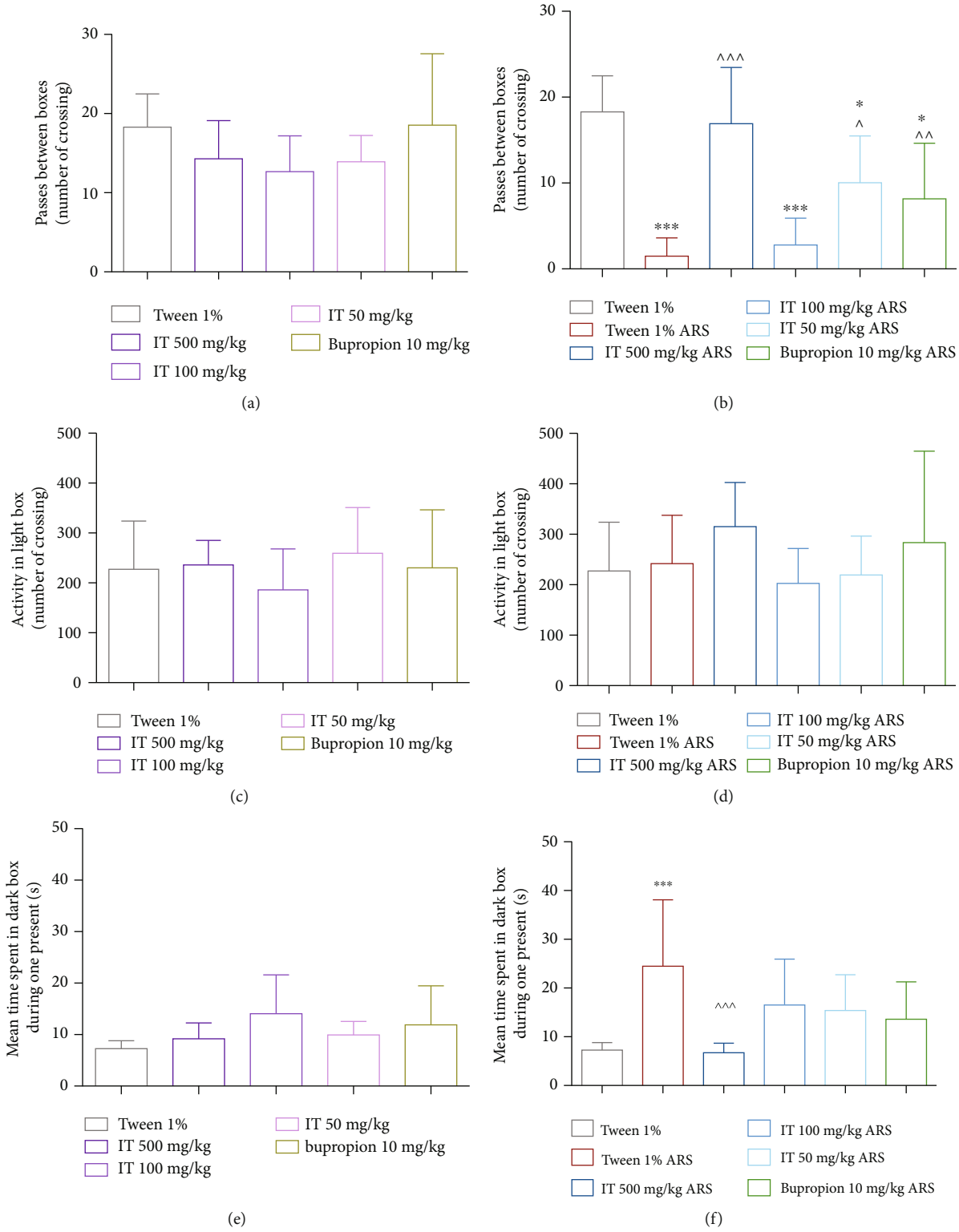

FIGURE 5: The effect of *Isatis tinctoria* leaf extract on behavior in an unknown space (dark/light box test). The number of passes through the hole between light and dark fields: (a) without stress (naïve mice), (b) after stress. Locomotor activity: (c) without stress (naïve mice), (d) after stress. The time spent by mice in the dark field of the activity cage during one stay: (e) without stress, (f) after stress. ARS: acute stress model, IT: *Isatis tinctoria*.

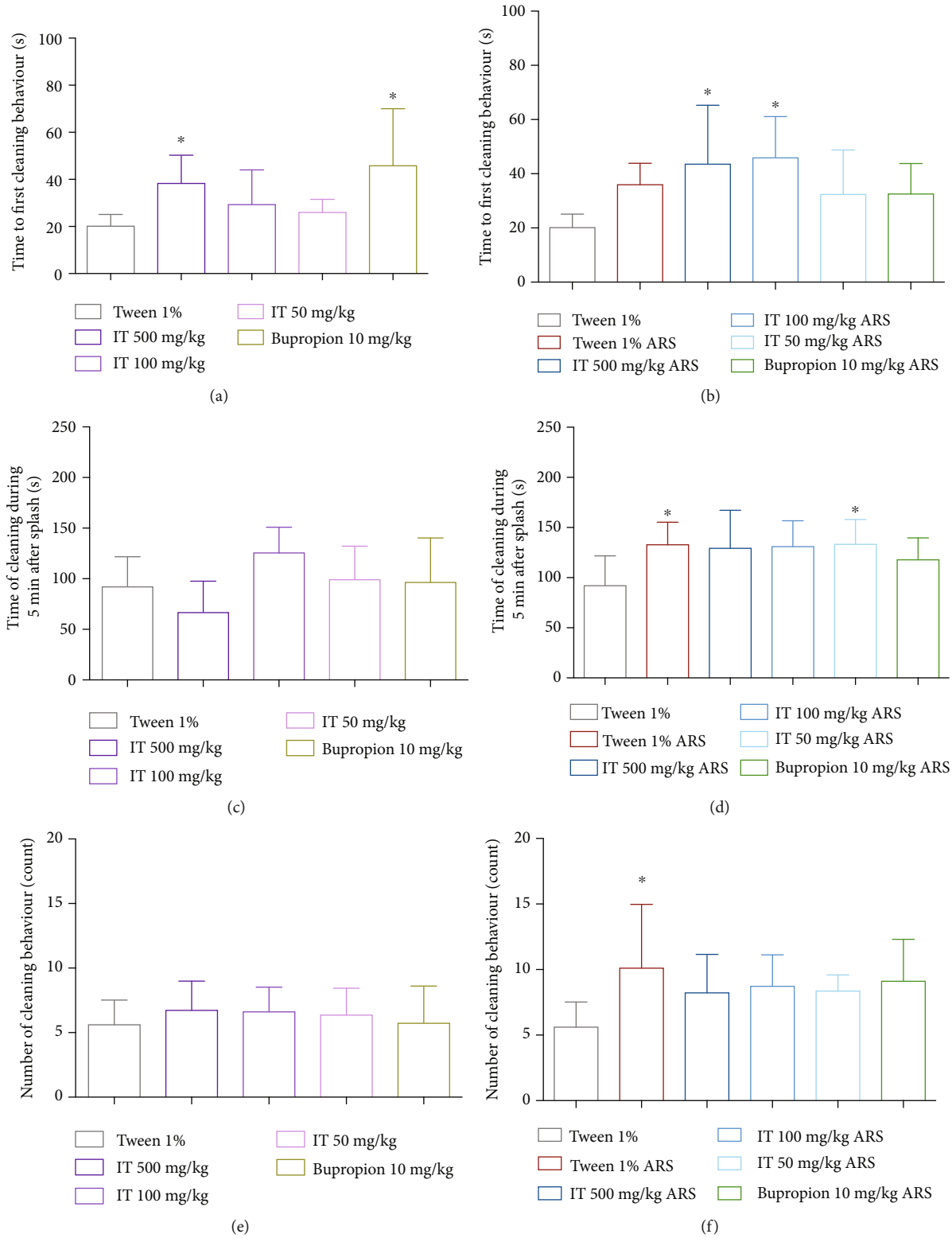

FIGURE 6: The effect of *Isatis tinctoria* leaf extract on behavior during splash test. The time to start washing by mice: (a) without stress (naïve mice), (b) after stress. The washing time of mice within 5 minutes: (c) without stress (naïve mice), (d) after stress. The number of washes: (e) without stress (naïve mice), (f) after stress. ARS: acute stress model, IT: *Isatis tinctoria*. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's or Tukey's post hoc test was used to calculate the significance of differences between the groups; $n = 7 - 8$. *Significant difference vs. the control group without stress; significance level: $^{*}p < 0.05$.

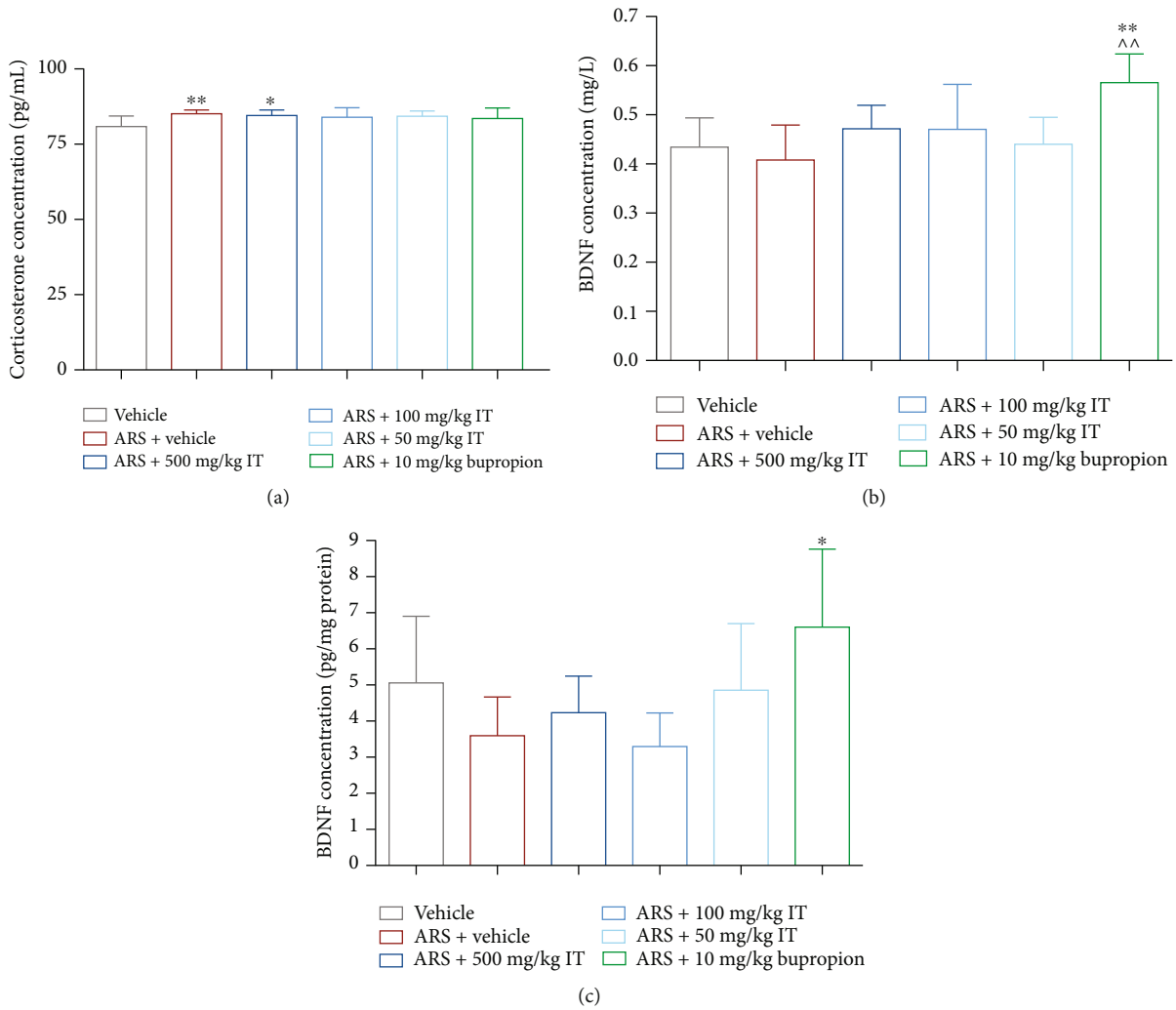

Figure 7: Levels of corticosterone or BDNF. (a) Plasma corticosterone, (b) plasma BDNF, and (c) brain BDNF (c). ARS: acute stress model, IT: *Isatis tinctoria*.

There were no differences in SOD activity in the brains collected from the different groups of animals. The results are shown in Figure 9(c).

The activity of CAT in the brain from the stressed control animals was significantly lower ($p < 0.05$) than the activity of this enzyme measured in the brain collected from the naïve control group. CAT activities after IT treatment at doses of 50 mg/kg b.w. or 500 mg/kg b.w. were significantly higher than SOD activity in the brain taken from the stressed control group ($p < 0.05$ and $p < 0.001$). After administration of the extract at a dose of 100 mg/kg b.w. or bupropion, the CAT activities determined in plasma did not differ significantly from the levels determined in the naïve control group. The results are shown in Figure 9(d).

The level of NO in the control brains taken from the stressed animals was significantly higher than in the naive control animals. In contrast, the levels of NO in stressed animals that were treated with IT were lower than in the brains of the stressed control group (they did not differ from the level determined in the naive control); in the case of the administration of the dose of 50 mg/kg b.w., statistical significance ($p < 0.05$) vs. the level of NO in the stressed control group was noted. Bupropion does not affect NO levels in the brain. In the brains of the bupropion-treated group, a comparable level of NO was detected as in the brains of the stressed control group. The results are shown in Figure 10(a).

There were no differences in TNF-alpha levels in brains collected from control and IT-treated animals. Significantly higher levels of TNF-alpha in comparison to both control groups were determined in the brain of mice treated with bupropion ($p < 0.001$). The results are shown in Figure 10(b).

4. Discussion

The present study shows for the first time that a single administration of hydroalcoholic leaf extract of *Isatis tinctoria* L. is able to reverse the behavioral response to restraint by a mechanism dependent, at least in part, on the modulation of oxidative stress, neuroinflammation, and NO reduction.

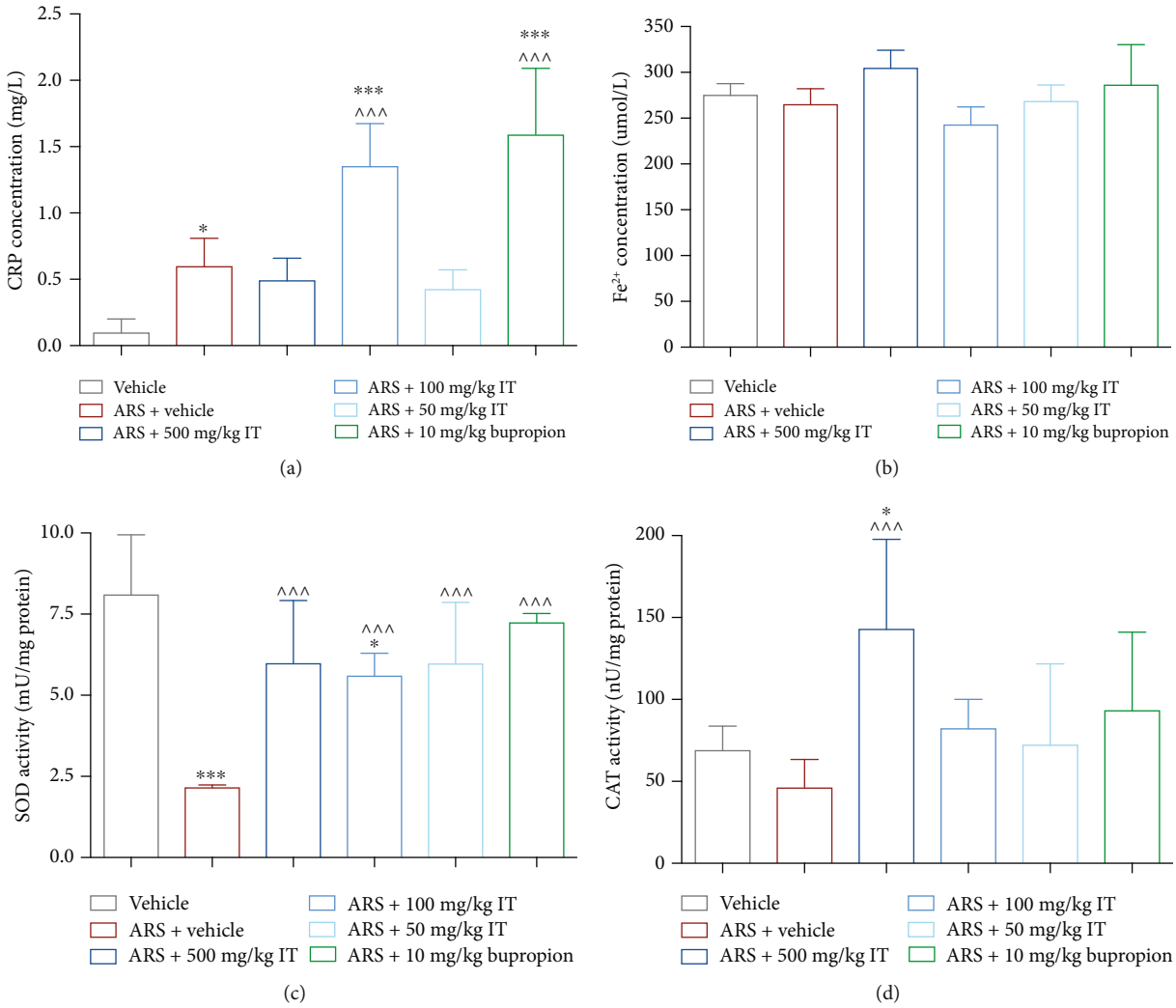

Figure 8: Plasma level of (a) CRP, (b) plasma total antioxidant activity, (c) plasma SOD activity, and (d) plasma CAT activity. ARS: acute stress model, IT: *Isatis tinctoria*. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Tukey's post hoc test was used to calculate the significance of differences between the groups; $n = 7 - 8$. *Significant difference vs. the control group without stress. ∧Significant difference vs. the control group with stress. Significance level: $^{*}p < 0.05$; $^{**,\wedge\wedge\wedge}p < 0.001$.

Isatis tinctoria is a plant with great therapeutic potential and extracts obtained from it possess the ability to inhibit the formation/activity of nitric oxide, anti-inflammatory, antioxidant, and neuroprotective properties [16, 39, 40]. Therefore, considering the above activities, preliminary pharmacological studies were performed to investigate the effect of polar extract from *I. tinctoria* leaves (IT) on stress-associated behaviors, as well as for the reduction of anxiety-like or depression-like behaviors. For this purpose, we used a validated model of acute restraint stress in mice [36, 41–43]. The latter is a widely used animal model to induce stress-related behaviors [36, 42], which are at least in part attributable to aberrations in the brain antioxidant and inflammatory systems [36, 44, 45].

Since most preclinical models target specific symptoms of psychiatric disorders [33], four behavioral tests were performed *in vivo*: the social interaction test, the dark/light box test, the splash test, and the locomotor activity test. These four behavioral tests are together suitable for phenotyping animal behavior that may be related to certain aspects of human behavior reflecting the behavioral aberrations in psychiatric conditions such as depression and anxiety [46].

Behavioral test results should always be interpreted considering the spontaneous activity of mice to ensure that the behavioral changes observed are not due to sedation or overstimulation after administration of the test extracts. Reducing spontaneous activity or overstimulation may contribute to false-positive or false-negative results. In this study, it was noticed that the intraperitoneal administration of IT at a dose of 50 mg/kg b.w. to nonstressed mice significantly reduces the spontaneous mobility of mice in comparison to the mobility determined in the control group, which indicates the sedative effect of the lowest dose of the extract tested. The plant extract is a mixture of various chemical

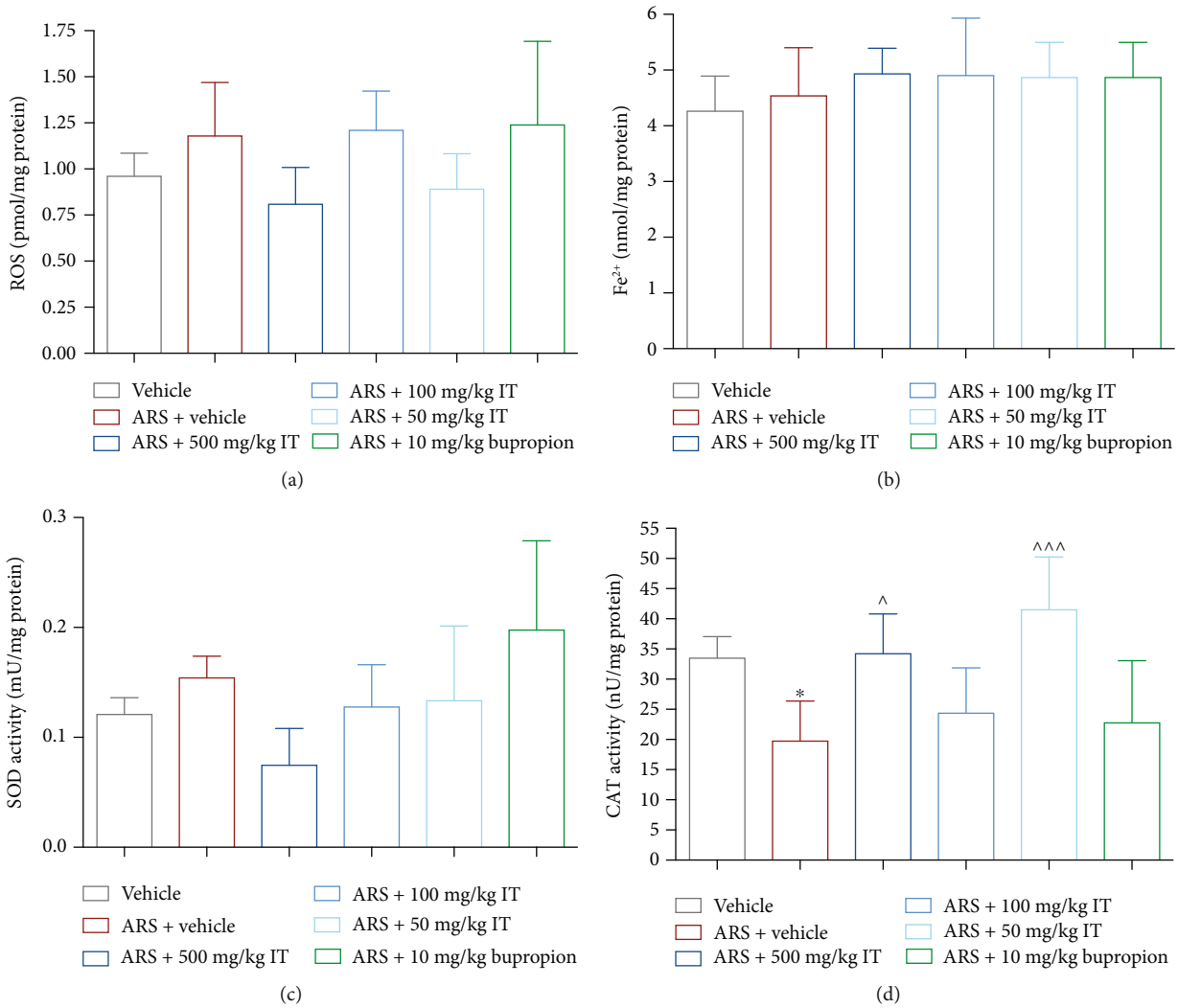

Figure 9: (a) Brian level of ROS, (b) brain total antioxidant activity, (c) brain SOD activity, and (d) brain CAT activity. ARS: acute stress model, IT: *Isatis tinctoria*. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Tukey's post hoc test was used to calculate the significance of differences between the groups; $n = 7 - 8$. *Significant difference vs. the control group without stress. ∧Significant difference vs. the control group with stress. Significance level: *,∧$p < 0.05$; **,∧∧∧$p < 0.001$.

compounds, and our research shows that in this lowest dose, we already have a sufficient presence of one ingredient to induce a significant sedative effect.

On the other hand, the lack of a sedative effect at higher doses may indicate that as the dose increases, the effects of other components of the extract cancel out this sedative effect. Further studies are planned to determine the specific compounds related to the effects detected in our research. However, a previous study [27] characterized the phenolic profile of polar extracts obtained from leaves of *I. tinctoria* by identifying Vicenin-2 and Isovitexin as the most abundant flavonoids. This analysis may suggest the hypothesis of the involvement of Vicenin-2 and Isovitexin in the activities reported by our experiment.

The downside is that the plant obtained from natural conditions may differ in composition depending on the substrate and the weather; therefore, after these preliminary tests with promising results, we plan to conduct research using the leaves of plants grown in the laboratory. If the biomass from in vitro cultures would match the level of plant activity, a material derived from in vitro cultures could be proposed as a homogeneous therapeutic material.

In the ARS model, stress caused a significant decrease in spontaneous mobility compared to the control group without stress. The mice, immobilized for 240 minutes, did not explore the activity cage to the same extent at the time of measurement as the unstressed mice. This may be due to, e.g., the immobilization-induced mental and physical fatigue and is a measurable symptom of induced stress. Administration of IT at a dose of 500 mg/kg b.w. in stressed mice significantly increased the activity of mice compared to stressed control mice. Thus, we can conclude that the highest tested dose of the extract, after which no significant effect on spontaneous mobility was observed in nonstressed mice,

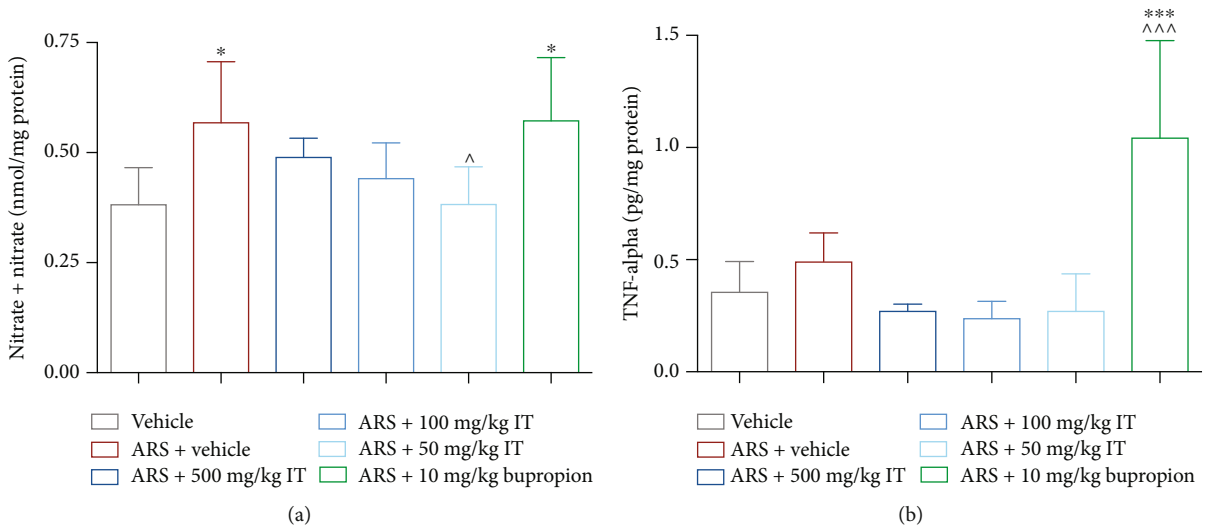

Figure 10: Levels of NO or TNF-alpha. (a) Brain NO, (b) brain TNF-alpha. ARS: acute stress model, IT: *Isatis tinctoria*. The results are presented as the mean of the measurements ± $\Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Tukey's post hoc test was used to calculate the significance of differences between the groups; $n = 7 - 8$. *Significant difference vs. the control group without stress. ∧Significant difference vs. the control group with stress. Significance level: *,∧$p < 0.05$; ***,∧∧∧$p < 0.001$.

abolished spontaneous mobility disorders caused by acute stress. It should be noted that after intraperitoneal administration of IT at a dose of 50 mg/kg b.w. in stressed mice, no statistically significant reduction in mobility was observed compared to the control group without stress, despite the earlier determination of a significant sedative effect of the extract at this dose in mice without stress. The sedative effect observed at this dose (seen in unstressed mice), therefore, might have made a minor contribution to the improved stress response in this regard in stressed mice. The reference compound selected for the study, bupropion, significantly increased the spontaneous mobility of mice in naïve mice, which is consistent with the known stimulating and activating effect of this drug [47]. It was found that after applying both these doses of IT or bupropion, the spontaneous activity in the stressed mice did not differ from the spontaneous activity counted in the control mice without stress. Therefore, we conclude that as for bupropion, it could have been due to its stimulating effect, while the tested extract at a dose of 500 mg/kg or 50 mg/kg b.w. had a positive effect on these poststress disorders.

Social behavior disorders are frequently observed in people suffering from affective disorders such as depression and anxiety states [33]. The social interaction test was carried out to identify aberrations in social interactions in our animal model study. This test is used to study the interaction between two unknown mice in an open field [33]. The increase in the number of contacts may be due to the hyperactive behavior of the mouse. On the other hand, the reduced number of contacts may be related to the depressive or anxious behavior of the animals [48] and also result from sedation; therefore, the results are interpreted considering the effect on spontaneous activity. In a social interaction study performed, the number of times the test mouse approached the stimulating mouse was analyzed. In non-stressed mice, the extract administered at all doses and the reference compound did not influence social behavior. Even after administration of the extract at a dose of 50 mg/kg b.w., which caused sedation, no significant changes in the interaction between mice were observed in this test. The curiosity of the second subject was therefore stronger than that of the sedative effect, which is indeed a favorable result. It was observed, however, that the number of contacts initiated by stressed control mice is statistically significantly lower compared to contacts initiated by stress-free control mice, thus indicating a significant role that stress plays in impaired social functions. All the doses of IT tested, as well as the reference compound, bupropion, increased the number of approaches of the test mice to the stimulant mice (no statistical significance was also observed compared to stress-free control mice; therefore, the effect should be considered noticeable), and bupropion showed this effect is strongest; statistical significance was calculated compared to the stressed control group. This is probably due to the stimulating and activating effect of bupropion [47].

The dark/light box test was used to initially assess the anxiolytic effect of the test extracts. It is based on the rodent's innate aversion to brightly lit, open areas (mice like to spend time in small, sheltered spaces), and spontaneous exploratory behavior of new environments (mice placed in a new environment learn about their surroundings out of curiosity) [34]. In times of stress, a smaller, dark box is a safer place for mice compared to a larger, illuminated chamber, which they are reluctant to explore in this state. The dark/light box test is quick and easy to perform and requires no prior training of the animals. The anxiolytic effect of the extracts tested is indicated by an increase in spontaneous activity of mice, a decrease in the time spent in the dark part of the cage, and an increase in the number of passes through the opening between the light and dark chambers [34].

Therefore, the conducted light/dark box test focused on the analysis of three parameters: the number of passes through the opening between the light and dark field, activity in the bright field of the locomotive cage, and the time spent in the dark field during one entry into this part of the chamber. It was observed that the number of passes through the opening in the stressed control mice was statistically significantly lower than in the nonstressed control group. These results were in line with the results indicating sedation after a period of stress in the previously discussed test of spontaneous activity (stress reduced this activity). This reduction in the number of passages between boxes also proves the dullness of curiosity to explore by these animals and the reduction of the need to explore new spaces or the feeling of poststress anxiety.

It was also noted that in the ARS model, the extract was administered at a dose of 100 mg/kg b.w. did not significantly affect the number of passes between boxes; their number was comparable to the number of passes in stressed control mice. Therefore, no beneficial effect was shown at this dose. Administration of IT in doses of 500 mg/kg b.w. and 50 mg/kg b.w. caused more frequent passes of mice between light and dark fields compared to stressed control mice, with the number of passes being greater after administration of the extract at the highest dose. These results indicate the anxiolytic properties of the IT administered in the highest and the lowest concentration tested. As previously emphasized, the extract at a dose of 50 mg/kg b.w. in nonstressed mice induced a significant reduction in activity; therefore, it should be noted once again that in stressed mice, this sedative effect may be beneficial, which can be seen in the reduction of stress symptoms. The lack of an intermediate dose effect in this test, therefore, it can be explained that in this dose, the effect of the sedative compounds contained in the extract is already canceled out by the greater presence of compounds with the opposite activity in the extract. However, the composition of this dose does not yet cause anxiolytic effects noticeable after administering the highest dose of the extract.

Measurement of locomotor activity of mice in the light box after administration of all tested doses of extracts did not provide statistically significant results. However, the videos with the recordings of the experiment were watched, and it is clearly visible that the mice subjected to stress explored the cage less and sat in one place more often. However, they also made a significant number of movements while cleaning the fur (they visibly sweated during the stress induction period), and probably, these movements were also read by an automatic measuring device that recorded spontaneous activity in a bright box. Therefore, we conclude that the results were disturbed by the increased sweating during stress induction in the selected model and cannot be rightly interpreted.

However, when examining the time spent by mice in the dark box per entry, it was noticed that stressed mice spent statistically significantly more time in the dark box than control mice without stress. This is probably because naturally, dark places are perceived by mice as safer and nonthreatening, and mice under stress preferred to protect themselves there rather than explore the unknown, open, bright part of the box. All tested doses of IT, as well as the reference compound, bupropion, reduced the time spent by mice in the dark box during one stay compared to the time spent by the stressed control group. However, only the extract administered at a dose of 500 mg/kg b.w. shortened this time in a statistically significant way. This is further evidence of the anxiolytic effect of the highest tested dose of IT.

The splash test is used to assess anhedonia, which is one of the symptoms of depression [49]. Spraying the hair on the back of mice with a 10% viscous solution of sucrose induces grooming behavior, and the lack of it is considered an analogy of anhedonia [49]. In this study, the time to start washing, the total washing time, and the number of washes were measured, that is, parameters described in the literature as related to motivational behavior and self-care [49]. The test shows, however, that the administration of the extract in the maximum dose of 500 mg/kg b.w. to nonstressed mice, as well as bupropion, significantly extended the time to start washing. These are surprising observations, but in the case of bupropion, which increased spontaneous mobility at such a dose, it could have been due to agitation and the desire to visit a new space for mice, in which it was placed immediately after spraying, i.e., at the initial time of the test. While these two results appear logically complementary, the limitation of the study performed was the lack of a concurrent measurement of mobility, a test that could accurately indicate the dependence of not initiating cleaning on increased activity. However, more research is needed to explain the observation of this parameter regarding the effect of IT at the highest dose used.

Surprising results were obtained with mice in the ARS model. It turned out that subjecting the mice to stress did not cause a statistically significant extension of the time to start washing compared to nonstressed control mice, and the administration of IT at all doses tested did not significantly affect this time compared to stressed control mice. When analyzing the washing time, it was observed that stressed mice washed longer than nonstressed mice. The counts of the number of washes also provided results that were different from what was expected. Thus, inducing stress in mice resulted in more frequent grooming behavior compared to the stress-free control group. However, since all stressed mice sweat very significantly after the stress induction period, it could have a significant disruptive effect on the parameters determined in this test. Therefore, it should be concluded that the splash test is not suitable for evaluating depressive behaviors such as anhedonia after stress induction by temporary immobilization. Therefore, there is a real need to conduct another test in the future that will give unambiguous results.

Keeping in mind the knowledge that physical and mental stress activates cellular stress by intracellular pathways involved in increasing free radical production [50–52], we determined the effect of stress caused by immobilization as well as the effect of a single administration of IT on the level of ROS in the brain and the activity of antioxidant enzymes: SOD and CAT in brain and plasma. Our research showed that under the stress levels of antioxidant enzyme activity

were significantly leveled after a single administration of IT. The most significant changes were observed after a dose of 500 mg/kg body weight and 50 mg/kg b.w., which correlates with the results of behavioral studies, because, as described above, it was the use of these doses that had a significant effect in reducing the symptoms of stress. It should be emphasized that the reduced CAT activity in the brain of stressed mice observed in the present study is in agreement with data previously reported by others [41, 43, 53]. Reduced CAT activity is an indicator of a prooxidative state since SOD converts superoxide anion to hydrogen peroxide (H_2O_2), but CAT does not metabolize H_2O_2 to water [54]. This excessive production of H_2O_2 may favor the Fenton reaction and the generation of hydroxyl radical, which in turn triggers lipid peroxidation. Therefore, we propose that one of the mechanisms of IT to reduce stress-induced behavior is to increase the ability to combat oxidative stress by influencing the activity of CAT (probably indirectly).

Nitric oxide is also known to modulate levels of cyclic guanosine monophosphate, which in turn induces a depression-like state in animals, reducing motivation to cope with stress [55]. In our study, we observed significantly increased levels of nitric oxide in the brain in stressed mice. However, what is worth emphasizing and may indicate a second mechanism of action in reducing poststress disorders, administration of IT reduced the level of NO in the brain, and it was best observed at the lowest dose used (50 mg/kg b.w.).

CRP is an acute phase protein that is widely used in clinical practice and has also been measured in many previous studies of behavioral disorders [56–58]. Additionally, it is known that elevated levels of TNF-alpha in the brain may be associated with local inflammatory pathways [56]. Recent data indicate that increasing plasma CRP concentrations in patients is also associated with decreased functional connectivity within reward circuitry and with high central nervous system glutamate level, which is correlated with symptoms of anhedonia [59]. In our study, we measured the level of CRP in the plasma taken from mice, as well as the level of TNF-alpha in the brains. In fact, it turned out that in stressed mice, plasma CRP levels increased significantly, but no significant changes in TNF-alpha levels were observed. These results may be because we performed an acute stress model, and the increased levels of TNF-alpha in the brain after this acute stress may not be visible enough yet. What is important, however, is the result obtained after administration of the IT, in doses reducing the symptoms of behavioral disorders, i.e., 500 and 50 mg/kg b.w., the level of CRP in the plasma was lower compared to the level determined in the stressed mice receiving only the vehicle (it did not differ from the level determined in unstressed mice). This underlines the importance of the anti-inflammatory effect of IT in reducing stress-induced disorders and may indicate a third mechanism of this action.

We used bupropion, an atypical antidepressant that inhibits the reuptake of noradrenaline and dopamine, as the reference compound in our research. It was effective in stress-initiated behavioral disorders (it had an activating and anxiolytic-like effect). Biochemical assays showed that bupropion also balanced the activity of antioxidant enzymes but did not affect the level of NO in the brain, and the marked markers of inflammation were even higher in the plasma and brain of the receiving group than in control animals. Bupropion increased the levels of BDNF in both the plasma and brain, which was not observed with IT. The stronger effect of reducing stress-induced behavioral disturbances after IT administration than after bupropion may therefore be the result of several mechanisms of action. Further extended studies are necessary to confirm and identify the specific target points of the action of the IT.

It is important to note that the *I. tinctoria* can be useful to treat stress-related disorders such as depression and anxiety and that this plant should be trialed in other conditions and disorders that are associated with immune-inflammatory and nitrooxidative stress processes and reduced neuroprotection. As such, extracts of this plant may be beneficial in preventing or treating suicidal behaviors [60], psychosis [61], mild cognitive impairment [62], and neurological disorders including Parkinson's and Alzheimer's diseases [63] and temporal lobe epilepsy [64].

5. Conclusion

These preliminary studies clearly show that *Isatis tinctoria* leaf extract may reduce stress-induced behavioral disturbances by regulating neurooxidative, neuronitrosative, and neuroimmune pathways. Therefore, extracts of this plant may be considered for further research on reducing stress-induced behavioral disorders. Confirmation of the beneficial properties of IT against stress-associated disorders is a first step and encourages research on plant material grown *in vitro* cultures, with a uniform and controlled metabolic profile, i.e., biochemically and/or genetically uniform biological material.

Data Availability

The data presented in this study are available on request from the corresponding author.

Ethical Approval

All applicable international laws for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Magdalena Kotańska conceived and designed the research. Noemi Nicosia, Magdalena Kotańska, Marek Bednarski, and Justyna Mazurek conducted the experiments and analyzed the data. Natalizia Miceli and Inga Kwiecień prepared

the extracts of plant. All authors contributed to the interpretation of the data and writing of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Figures 1 and 2 were created with BioRender.com.

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Retraction

Retracted: Carveol Promotes Nrf2 Contribution in Depressive Disorders through an Anti-inflammatory Mechanism

Oxidative Medicine and Cellular Longevity

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
(4) Inappropriate citations
(5) Incoherent, meaningless and/or irrelevant content included in the article
(6) Peer-review manipulation

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] A. J. Muhammad, L. Hao, L. T. Al Kury et al., "Carveol Promotes Nrf2 Contribution in Depressive Disorders through an Anti-inflammatory Mechanism," *Oxidative Medicine and Cellular Longevity*, vol. 2022, Article ID 4509204, 16 pages, 2022.

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Research Article

Carveol Promotes Nrf2 Contribution in Depressive Disorders through an Anti-inflammatory Mechanism

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Major depressive disorder (MDD) is a progressive deteriorating mental state with a feeling of worthlessness and frequent mood swings. Several studies reported the favorable effects of natural drug substances on MMD associated oxidative stress and neuroinflammation. The present study is attempted to examine whether carveol could affect lipopolysaccharide- (LPS-) induced depression, and if so, how nuclear factor E2-related factor (Nrf2) contributed to the neuroprotective effects of carveol mechanistically. Two experimental cohorts were used using the SD rats: first to evaluate the promising dose of carveol (whether 20 mg/kg or 50 mg/kg) and secondly to determine the effect of carveol on Nrf2-mediated antidepression. Significant neuronal alterations were noticed in the cortex and hippocampus regions in the LPS-treated group, accompanied by elevated inflammatory cytokine levels such as tumor necrosis factor-alpha (TNF-α), cyclooxygenase (COX-2), and c-Jun N-terminal kinase (p-JNK). Moreover, amassing of free radicals exacerbated lipid peroxidase (LPO) and oxidative stress with a limited antioxidant capacity. Carveol (20 mg/kg) significantly ameliorated these detrimental effects by promoting the antioxidant Nrf2 gene and protein, which critically regulate the downstream antioxidant and anti-inflammatory pathway. To further elaborate our hypothesis, we employed all-trans retinoic acid (ATRA), an Nrf2 inhibitor, and we found that ATRA exaggerated LPS-induced depressive-like effects associated with elevated neuroinflammatory markers. Our results demonstrated that carveol (20 mg/kg) could activate the endogenous antioxidant Nrf2, which regulates the downstream antioxidant signaling pathway, eventually leading to amelioration of LPS-induced neuroinflammation and neurodegeneration.

1. Introduction

Depressive disorders like major depression or MDD are the leading human problem with multifactorial abnormalities ranging from mood, emotion, and cognitive deficits along with recurrent thoughts of suicide [1, 2]. Depression is increasingly becoming a social and economic problem that costs billions of dollars [3], and up to 60% of suicidal cases could be linked to depressive-like symptoms [4]. Although, depression has been declared as a major contributor to the total burden of world

diseases by WHO [5], very little is known about the exact etiological cause and underlying pathophysiology. Moreover, several conditions such as stress exposure, metabolic and hormonal disorders, and drug addiction can precipitate the symptoms. Serotonin and/or norepinephrine-based drugs are extensively used so far, but the effectiveness of such antidepressant drugs is shrinking due to the unpredictable responses and their low recovery ratio. Therefore, looking into the risk-benefit ratio, subsequent alternatives are the demand of the day [6]. Furthermore, the complex mechanisms of existing antidepressant therapy along with poor prognosis aid in poor compliance. It is therefore need of the time to unveil alternative strategies to develop novel approaches for this purpose [7].

Neuroinflammation is involved in potentiating the severity of MDD, which is consistently reiterated in the literature [8, 9]. The surge in inflammatory mediators and cytokines cause the penetration of macrophages into the brain, validating the macrophage theory of depression [10]. Similarly, other research studies also implicated the role of inflammatory cascades in the pathophysiology of depression in both laboratory animals and meta-analysis of postmortem brain tissue samples [11, 12]. Furthermore, treatment with conventional antidepressants such as selective serotonin reuptake inhibitors (SSRIs) can reverse the elevated level of cytokine in addition to favorable outcomes on depression [13, 14]. However, other non-SSRIs can be used for the management of depression with no impact on cytokines [15]. This discrepancy is attributed to the heterogenetic nature of depressive disorders as MDD triggers proinflammatory cytokines [16, 17]. Maes et al. described inflammation as vital in propagating depression pathogenesis [18] as inflammatory cytokines trigger behavioral and cognitive deficits [19, 20], impaired neurotransmitter metabolism, and decrease neuroplasticity [21, 22]. Furthermore, the administration of lipopolysaccharide (LPS) can induce behavioral alterations in animals similar to that in humans [23, 24]. Based on these shreds of evidence, we used a well-studied inflammation inducer, lipopolysaccharide (LPS) to induce neuroinflammation, neurodegeneration, and behavioral deficits and thereby use it as a model of anxiety and depression in rodents [25–27].

It is well known that the cellular defense mechanism of the body involves the endogenous transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) against inflammation and oxidative stress. Nrf2 and Nrf2-mediated phase-II antioxidant enzymes have been well studied for their therapeutic role in the treatment of various neuronal disorders [28]. Furthermore, the contribution of Nrf2 in inflammation or inflammation-induced disorders such as stroke and other disorders is well documented in previous studies [29–31]. Therefore, dysregulation of the Nrf2 signaling pathway may cause increased susceptibility of the tissue to detrimental effects of oxidative stress and inflammatory mediators [32, 33]. Moreover, several research bodies have reported the neurotherapeutic role of Nrf2 and its downstream signaling in animal models of various neurological disorders [34]. Previously, it has been demonstrated that Nrf2 plays a crucial role in the pathophysiology of depression via regulating oxidative stress and inflammatory processes [35, 36]. Hence, targeting Nrf2 might be considered one of the potential pharmacological approaches for the inquisition of depressive behaviors.

Recently, studies suggest that food and nutritional supplement can modulate depressive-like symptoms [37–41], further supported by human meta-analyses [42, 43]. In line with these studies, natural moieties based on their rich antioxidant potential are frequently investigated as these drugs have therapeutic potential against many stress mediators, including inflammatory factors and free radical species [44]. Carveol, essential oil has previously demonstrated antidiabetic potential possibly by attenuating oxidative stress [45]. Furthermore, carveol has shown neuroprotective potential in the ischemic brain injury model by ameliorating the infarction area by promoting the Nrf2 pathway [33]. Also, carveol also demonstrated attenuation of memory impairment and behavioral deficits in rodent model possibly by ameliorating oxidative stress [46]. In another study, Zhang et al. reviewed that carveol and similar other natural oils mitigate depression-like symptoms [47]. Recently, we demonstrated that carveol attenuated acetaminophen-induced liver toxicity by modulating Nrf2 cascade and inflammatory cytokines [48]. Taking into account the pharmacological value of natural essential oils and the search for alternate drug therapy for depressive behaviors, the current study was designed to investigate the potential role of carveol against LPS-induced behavioral deficits, neuroinflammatory signals, and neurodegeneration in an animal model of rodents. Herein, we for the first time showed that carveol mediates its antidepressant and anxiolytic effect by regulating Nrf2 and its downstream inflammatory cascades.

2. Material and Methods

2.1. Chemicals and Reagents. All the chemicals and drugs such as carveol catalog number (192384-10G), all-trans retinoic acid (ATRA) trichloroacetic acid (TCA), 5,5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione (GSH), 1-Chloro-2,4-dinitrobenzene (CDNP), LPS, N-(1-Naphthyl) ethylenediamine dihydrochloride, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ABC Kit and primary antibodies were purchased from Santa Cruz Biotechnology such as an anti-HO-1 monoclonal antibody (mouse source, SC-136960), anti-Nrf2 (rabbit polyclonal, SC-722), anti-p-JNK (mouse monoclonal, SC-6254), anti-Bcl-2 (mouse monoclonal, sc-7382), and anti-COX-2 (mouse monoclonal, SC-514489). The secondary antibody (ab-6789) was purchased from Abcam (UK). The ELISA kits for Nrf2 (Cat # SU-B30429) and p-NF-κB (Cat # SUB28069) were purchased from China (Shanghai Yuchun Biotechnology), and HO-1 (Cat # E-EL-R0488) and TNF-α (Cat # E-EL-R0019) ELISA kits were purchased from Elabscience.

2.2. Animals and Drug Treatment. The male Sprague Dawley rats having average body weight ranging from 180 to 200 g were kept in a condition of a 12-hour light and dark cycle with access to water and food in the animal house facility. The animals were provided standard temperature and humidity according to the standard laboratory protocols, similar to the ARRIVE guidelines. All the experiments were performed according to the standard protocols, Riphah

Institute of Pharmaceutical Sciences (RIPS), Islamabad (Reference No: REC/RIPS/2020/07). The animals were acclimatized with the animal house facility before experimental procedures. The body weights were measured throughout the experimental period. The animals were divided into two experimental groups (a total of 80 animals) as follows (Figure 1).

2.3. Experimental Cohort 1. The first cohort was used to determine the effective dose of carveol ($n = 10$ animals/group) and consisted of saline, LPS, two carveol treatment groups with LPS as carveol 20 mg/kg (CAR 20) and carveol 50 mg/kg (CAR 50), and fluoxetine (5 mg/kg) with LPS. Saline, carveol, and fluoxetine were administered for 5 days as a single intraperitoneal dose (i.p), while LPS was administered on the 3rd and 4th day of the regimen either as a single dose or after carveol at a dose of 1 mg/kg, i.p, and fluoxetine injection as our previously reported data [27].

2.4. Experimental Cohort 2. The second cohort was used to determine the Nrf2 role and included three subgroupings ($n = 10$/group): ATRA in combination with LPS, carveol in combination with the ATRA + LPS group, and fluoxetine in combination with ATRA + LPS. ATRA was injected at least 30 min before LPS (i.p.).

2.5. Behavioral Studies. A gap period of at least 2 days was mentioned between each behavioral study.

2.5.1. Sucrose Splash Test (SST). The measurements were performed according to a standardized protocol as previously discussed [27]. The SST test was performed by spraying sucrose, and the typical parameters were recorded for 5 min, including licking and scraping the body to wash away the solution.

2.5.2. Elevated Plus-Maze (EPM) Test. The LPS-mediated anxiety was measured by an EPM test using a framework located about 50 cm above the floor. The EPM test was performed by keeping each rat in the middle of the platform, and the number of entries and time spent was recorded [49].

2.5.3. Light-Dark Box (LDB) Test. For the evaluation of anxiety-like behavior, the LDB test was performed using the customized light-dark box. The LDB is composed of light and dark compartments partitioned by a small gap or entry point. The test was performed by placing each experimental animal in the dark compartment of the light-dark box and was allowed to move freely in the box for 5 min. The data was recorded (videotaped), and the total number of entries in each compartment was noted. After completion of each test, the box was cleaned using alcohol to minimize the olfactory cues [50].

2.5.4. Forced Swim Test (FST). The forced swim test was performed to evaluate the depression level in rats. The rat was placed in a Plexiglas cylinder which was 70 cm in height and 30 cm in diameter, at a specific temperature of 23 ± 1 °C. A preswim exposure test was performed 24 h before the test to delineate the antidepressant-like activity. The use of a preswim ensures that the rats quickly adopt an immobile posture on the test day, which enables the effect of the tested compounds to be more easily observed. The test was performed by filling the cylinder with water (above 30 cm height) at 23 ± 1°C temperature and videotaped for 7 min. The final 4 min time of the test was randomly assessed at 5 s intervals for various parameters such as immobility.

2.6. Antioxidant Assays

2.6.1. Determination of Lipid Peroxidation (LPO). This assay was performed according to our previously established laboratory protocol [51]. The tissue was homogenized in lysis buffer. The tissue homogenate was centrifuged, and the supernatant was further mixed with freshly prepared ammonium sulphate solution. After the addition of TBA, the absorbance was recorded at 532 nm.

2.6.2. Reduced Glutathione (GSH) Level. The GSH assay was performed as previously described [52]. The 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) 0.6 mM was added to the 6.6 μL sample, and the GSH level was measured as described previously [52]. The absorbance was recorded at 412 (nm) wavelength using a microplate reader.

2.6.3. Glutathione S-Transferase (GST) Activity. The GST assay was performed for the activity of GST using the 1-Chloro-2,4-dinitrobenzene (CDNB) as a substrate as previously reported [53]. The assay protocol includes that each well was filled with 10 μL of 1 mM CDNB, 10 μL of 5 mM reduced glutathione, 270 μL of buffer solution, and 10 μL of the sample. The absorbance was read at 340 nm using a plate reader.

2.6.4. Catalase Activity. The catalase assay was performed by mixing H_2O_2 and the tissue supernatant. The absorbance was measured at 240 nm wavelength compared with the blank containing PBS only. The absorbance or catalase activity is proportional to the degradation of H_2O_2 to its desired product. So actually, the assay determines the breakdown of H_2O_2 so the results are expressed as μmol H_2O_2 decomposed per mg of protein/min [54].

2.7. Histological Preparation. The rats after behavioral analysis were decapitated, and the brain was removed. The brain was fixed, and the blocks were made. After that, we made three mm thick sections using a sharp blade and fixed in a 4% paraformaldehyde solution. The tissues were embedded into paraffin blocks and trimmed to 4 μm thin coronal sections using a microtome, and the following staining techniques were applied.

2.8. Hematoxylin and Eosin (H&E) Staining. Starting from dewaxing/deparaffinization and then rehydration step using a gradient alcohol series, which was ended by rinsing slides in distilled water, slides were stained with hematoxylin and eosin as discussed [55]. Finally, slides were dehydrated, and color was fixed in xylene and observed by a light microscope (Olympus, Japan).

2.9. Immunohistochemical Analysis. We employed a previously described procedure with slight modifications for immunohistochemical analysis [56]. After completion of

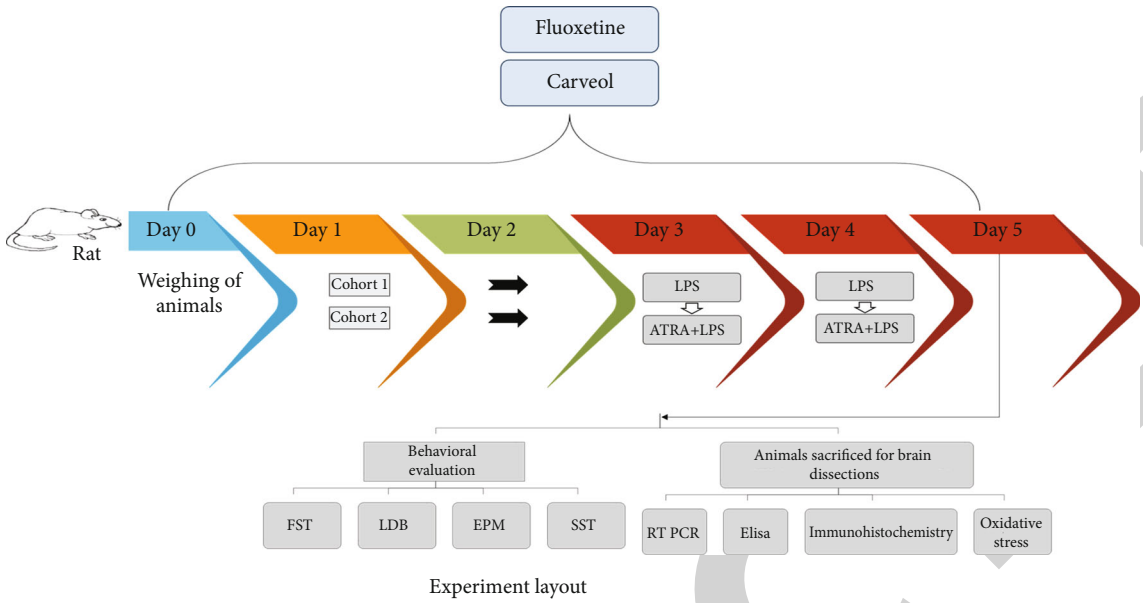


Figure 1: Experimental outline.

the deparaffinization step, slides were processed by an enzymatic method for antigen retrieval and then washed with PBS consecutively three times for 5 min. Slides were immersed in 3% H_2O_2 to quench endogenous peroxidase activity followed by washing with PBS. Normal goat serum (5%) was applied as a blocking serum, and slides were incubated for 2 h. Next, the slides were incubated overnight with primary antibodies Bcl2, p-JNK, TNF-α, Nrf2, HO-1, and COX-2. The next morning, slides were washed with PBS and incubated for 90 min with the secondary antibody, then incubated with an ABC kit (Santa Cruz) in a humidified box for 60 min. Slides were then washed with PBS solution and stained with DAB, followed by dehydration with ethanol (70%, 80%, 90%, and 100%). After dehydration, slides were fixed with xylene and then cover slipped with mounting media. Images were obtained using a light microscope and saved in TIFF format for further quantification by the ImageJ software.

2.10. ELISA Analysis. 50-70 mg of cortical and hippocampal brain tissue was first homogenized and then centrifuged at 15,000 rpm at 4°C to carefully collect the supernatant while avoiding the pellet. ELISA procedures were performed according to the manufacturer protocols using an ELISA microplate reader (BioTek ELx808), and the concentration (pg/mL) was then normalized to the total protein content (pg/mg total protein).

2.11. Real-Time Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from the rat cortical tissue in TRIzol as discussed previously [57]. 20 μL of M-MuLV reverse transcriptase was used to dilute 1 microgram of RNA and used this mix to synthesize cDNA with a cDNA synthesis kit (vivantis cDSK01-050 Sdn. Bhd, Malaysia). To estimate the gene expression of *Nrf2* quantitatively, real-time PCR was performed using the 2X HOT SYBR Green qPCR master mix (Solar Bio cat # SR1110) and real-time Mic PCR (Bio-Molecular System) according to the manufacturer specifications. The sequence of the primers used for amplification was *Nrf2*, Forward: CACATCCAGACAGACACCAGT and Reverse: CTACAAATGGGAATGTCTCTGC; *HO-1*, Forward: CGTGCAGAGAATTCTGAGTTC and Reverse AGACGCTTTACGTAGTGCTG; and GAPDH, Forward: AGGTCGGTGTGAACGGATTTG and Reverse: TGTAGA CCATGTAGTTGAGGTCA. The relative gene expressions of *Nrf2* were determined by the 2^-$\Delta\Delta$CT method for real-time quantitative PCR.

2.12. Statistical Analysis. Data were analyzed using GraphPad Prism and were expressed as mean ± standard error of the mean (SEM). Data were further analyzed by one-way ANOVA while using post hoc as Bonferroni multiple comparisons. $p < 0.05$ was considered significant. The symbol $*$ shows a significant difference relative to the saline group, and # shows a significant difference relative to the LPS group, while † represents a significant difference to ATRA + LPS.

3. Results

3.1. Carveol Attenuated LPS-Induced Depression-Like Behavior. LPS-treatment induced depression-like behavior as shown by reduced struggling and by immobile nature in the behavioral FST (Figure 2(a), $**p < 0.01$), coexisting with anxiety-like behavior as entries to the open arms were little or absent in the EPM test and also the time spent in the open arms was short suggesting less exploration compared to the control group (Figure 2(b), $**p < 0.01$). In accordance, LPS caused a greater stay in the dark compartment in the LDB test (Figure 2(c), $*** p < 0.001$), while significantly decreased grooming time in SST (Figure 2(d), $*** p < 0.001$). Carveol treatment with 20 mg/kg dose reversed all these behavioral

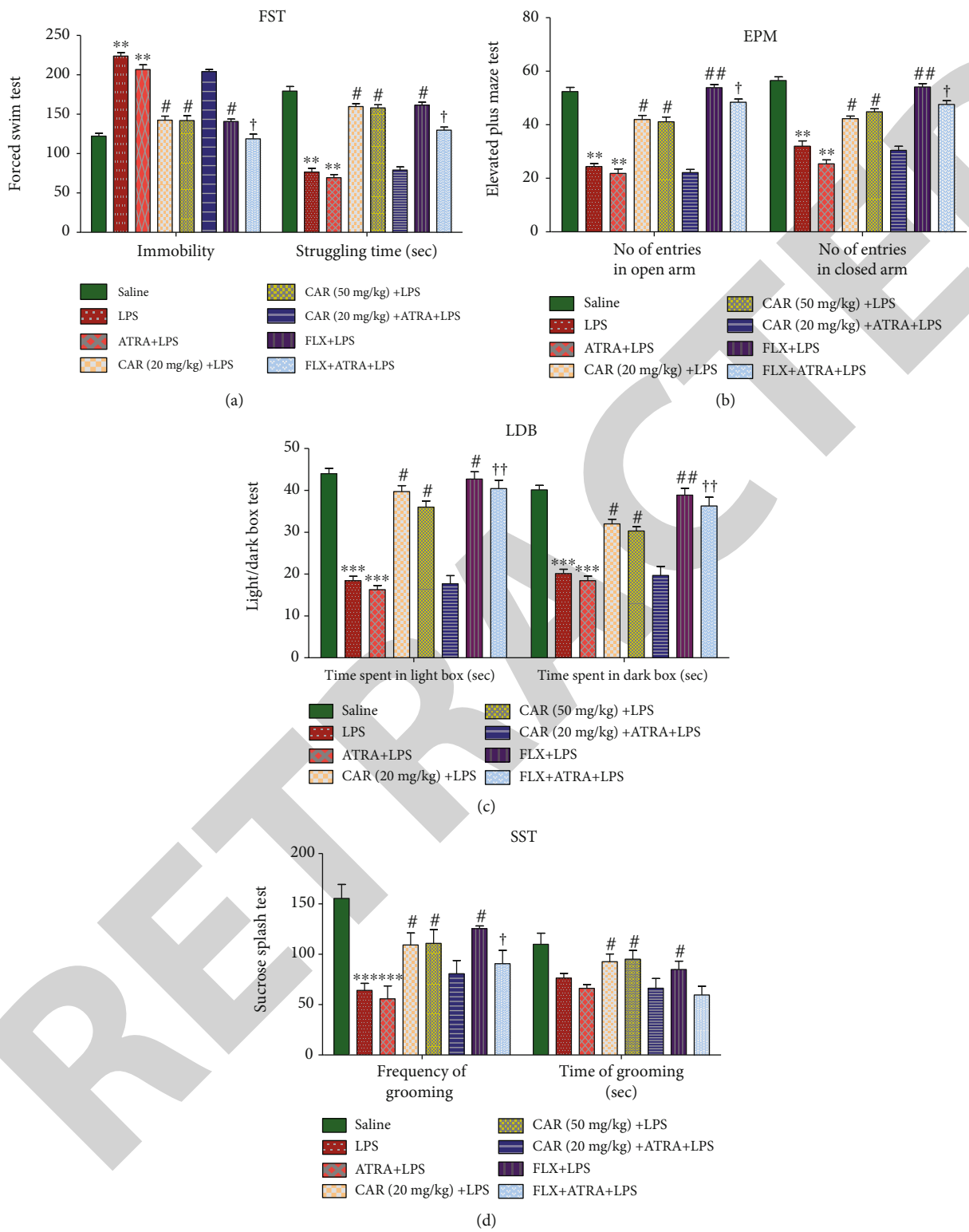


Figure 2: Effects of carveol (CAR) on LPS-induced behavioral deficits. Effect of CAR and LPS on the FST test (a), EPM (b), LDB (c), and SST (d). Data are expressed as means ± SEM and analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test using the GraphPad Prism 6 software. The saline, LPS, CAR+LPS, and FLX + LPS groups represent the first cohort ($n = 10$/group), while the ATRA+LPS, CAR +ATRA+LPS, and ATRA+LPS + FLX were from the second cohort ($n = 10$/group). $^{**}p < 0.01$ and $^{***}p < 0.01$ are compared to the saline group; $^{\#\#}p < 0.01$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group. $^{\dagger}p < 0.05$ is compared to ATRA+LPS. CAR: carveol; LPS: lipopolysaccharide; ATRA: all-trans retinoic acid; FLX: fluoxetine; EPM: elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test.

alterations in the FST (Figure 2(a), $^{\#}p < 0.05$) and in the EPM test (Figure 2(b), $^{\#}p < 0.05$), increased time spent in light compartment in LDB (Figure 2(c), $^{\#}p < 0.05$), and increased the grooming time (Figure 2(d), $^{\#}p < 0.05$) in SST. Similarly, treatment with carveol (50 mg/kg) produces the same effects in all these behavioral sets. Furthermore, cotreatment of LPS with ATRA exacerbated the anxiety-like behavioral deficits, while administration of carveol in this second cohort study did not elicit any ameliorative effects on LPS-induced depression-like behavior in the ATRA-treated groups.

3.2. Carveol Reversed LPS-Induced Cellular Damage. We performed HE staining to determine the rate and extent of neuronal cell death in response to LPS treatment, and we observed significant variations in neuronal shape and size ($*** p < 0.001$, Figure 3), compared to the saline group. The saline group exhibited normal morphological architecture of cell shape with no change in color staining, and there was no vacuole formation (Figure 3). Moreover, multiple vacuoles were noted in the LPS-treated groups associated with inflammatory infiltrated cells. Carveol administration mitigated these changes, and a higher degree of cellular integrity was evident in the carveol- (20 mg/kg-) treated group (Figure 3, $^{\#\#}p < 0.01$). Furthermore, coadministration of LPS and ATRA exacerbated the histopathological changes while pretreatment of carveol in the ATRA-treated groups did not show any significant alteration in the LPS-induced histopathological changes.

3.3. Carveol Attenuated LPS-Induced Apoptosis Markers. To further evaluate the antiapoptotic properties of carveol, we performed immunohistochemistry analysis for antiapoptotic factor Bcl-2 and apoptosis-linked p-JNK (Figure 4(a)). The expression of the Bcl-2 protein was decreased (Figure 4(a), $**p < 0.01$), coexisting with an upregulated p-JNK level in the LPS-treated group (Figure 4(b), $**p < 0.01$). However, treatment with carveol reverts these changes in the LPS group. Moreover, carveol pretreatment did not attenuate the LPS-induced apoptosis in the cortex of the ATRA-treated groups.

3.4. Carveol Augmented the Antioxidant Potential of the Brain by Promoting the Nrf2 Signaling Pathway. The mechanism of antioxidant capacity of carveol was determined by investigating the *Nrf2 gene* and Nrf2 protein and its downstream signaling *HO-1* gene and protein. Real-time PCR analysis showed that LPS treatment significantly reduced the expression level of *Nrf2* and *HO-1* compared to that of the control group (Figure 5(a), $*p < 0.05$). To further validate, we performed ELISA (Figure 5(b), $*p < 0.05$) and immunohistochemistry analysis (Figure 5(c), $**p < 0.01$), and consistent results were obtained. Likely, the downregulated proteins of Nrf2, antioxidant protein HO-1 expression was also significantly attenuated in the cortex and hippocampus of the LPS-treated group compared to that of the saline group (Figures 5(d)–5(f)). Administration of carveol along with LPS, induced upregulation of *Nrf2* and *HO-1* genes relative to that of the LPS group (Figures 5(a) and 5(d), $^{\#\#}p < 0.01$). Furthermore, noticeably elevated levels of Nrf2 and HO-1 were also observed using ELISA and immunohistochemistry (Figures 5(b), 5(c), 5(e), and 5(f)). However, the carveol-mediated upregulation of the antioxidant proteins Nrf2 or HO-1 was not observed in the ATRA-treated groups. These results suggest that carveol might possess potential antioxidant activity via activation of the Nrf2 and its downstream proteins like HO-1.

3.5. Carveol Inhibits LPS-Induced Neuroinflammation. The role of inflammatory mediators in depression studies is well documented; therefore, we also sought to investigate whether carveol treatment can be effective against neuroinflammation. We studied the protein expression of inflammatory markers such as p-NFkB, COX-2, and TNF-α using ELISA and immunohistochemistry analysis. Our results showed that the levels of p-NFkB, TNF-α, and COX-2 were significantly increased in the LPS group compared to that of the saline group (Figures 6(a)–6(c)). Pretreatment of carveol induced a marked downregulation of p-NFkB (Figure 6(a), $^{\#}p < 0.05$) and TNF-α (Figure 6(b), $^{\#}p < 0.05$) in the cortex and COX-2 (Figure 6(c), $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$) in the cortex and hippocampus relative to the LPS-treated group. Furthermore, cotreatment of LPS with ATRA further exaggerated the level of neuroinflammatory mediators, while carveol administration to the LPS- and ATRA-treated group did not show any protective effect against the detrimental effects of LPS and ATRA treatment.

3.6. Effects of Carveol Pretreatment on LPS-Induced Lipid Peroxidation and Antioxidant Enzymes. To investigate the neuroprotective and antioxidant activity of carveol, we measured the levels of various enzymes such as catalase, GST, GSH, and thiobarbituric acid reactive substances (TBARS) in both the cortex and hippocampus. Our results showed that carveol administration significantly ameliorated the LPS-induced oxidative stress via restoring the expression of the antioxidant enzymes in both the cortex and hippocampus. The levels of catalase, GST, and GSH were significantly lowered in the LPS-treated group compared to that of the saline group (Figures 7(a)–7(f), $*** p < 0.001$ and $**p < 0.01$). In accordance, a marked elevation in the level of TBARS was observed in the LPS-treated brain compared to that of the saline group (Figures 7(g) and 7(h), $**p < 0.01$). Carveol administration induced the production of the antioxidant enzymes, i.e., catalase, GST, and GSH, in both the prefrontal cortex and hippocampus (Figures 7(a) and 7(b), $^{\#\#}p < 0.01$; Figures 7(c) and 7(d), $^{\#}p < 0.05$ and Figures 7(e) and 7(f), $^{\#}p < 0.05$). On the other hand, a noticeably decreased level of TBARS was observed in the carveol-treated group as compared to that of the LPS-treated group (Figures 7(g) and 7(h), $\#p < 0.05$). Furthermore, carveol administration in the ATRA-treated groups did not show any antioxidant activity (Figure 7), validating the results of endogenous antioxidant enzyme Nrf2 (Figure 5). Finally, our results revealed that carveol might possess potential free radical scavenging activity to attenuate the LPS-induced oxidative stress in the cortex and hippocampus of a rat's brain.

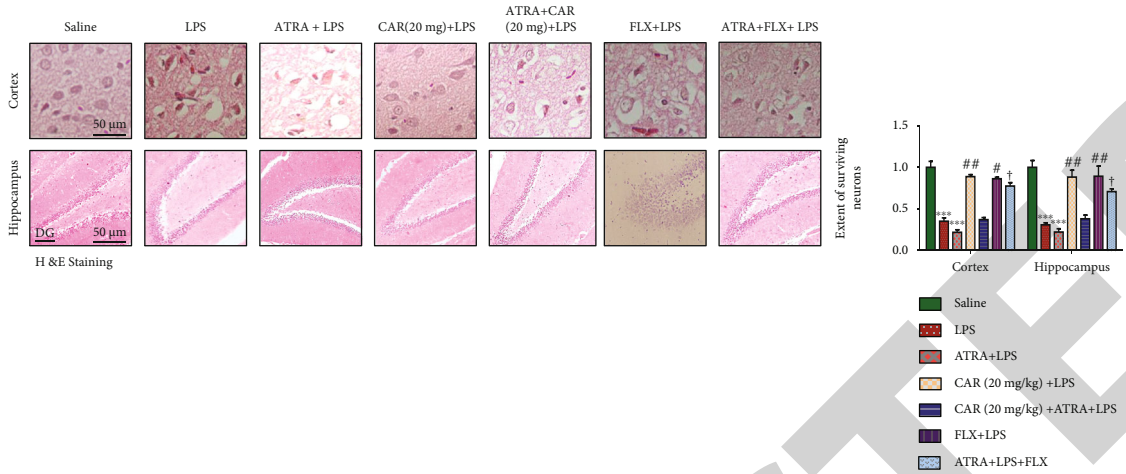


Figure 3: Hematoxylin and eosin (H&E) staining showing the extent of surviving neurons in the cortex and hippocampus (*Corno amonus, CA*). Scale bar 50 μm, magnification 40x. Dead neurons were characterized by a swollen cytoplasm, vacuolization, scalloped morphology with intense cytoplasmic eosinophilia, and nuclear basophilia. Data are expressed as means ± SEM. $^{***}p < 0.001$ is compared to the saline group; $^{\#\#}p < 0.01$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group; $^{\dagger}p < 0.05$ is compared to ATRA+LPS. CAR 20: carveol (20 mg/kg); LPS: lipopolysaccharide; ATRA: all-trans retinoic acid; FLX: fluoxetine. The H&E slides were made after the euthanization of animals following behavioral analysis. The saline, LPS, CAR+LPS, and FLX + LPS groups were those studied in the first cohort ($n = 5$/group), while the ATRA+LPS, CAR+ATRA+LPS, and ATRA+LPS + FLX groups were from the second cohort ($n = 5$/group).

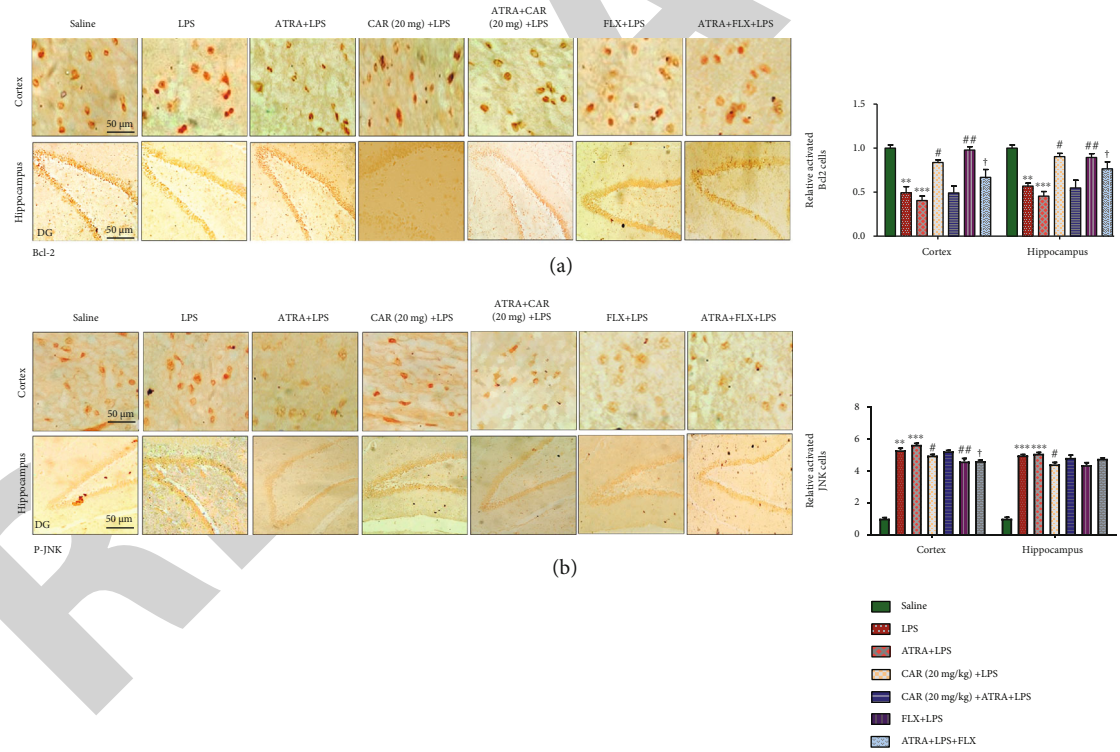


Figure 4: Effects of carveol on LPS-induced neuronal apoptosis. (a) Immunohistochemistry results for Bcl-2 in the cortex and hippocampus. Scale bar 50 μm, magnification 40x. Data are expressed as means ± SEM. $^{***}p < 0.001$ and $^{**}p < 0.01$ are compared to the saline group; $\#\#p < 0.01$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group, while $^{\dagger}p < 0.05$ is compared to ATRA+LPS. (b) Immunohistochemistry results for p-JNK in the cortex and hippocampus. Scale bar 50 μm, magnification 40x. Data are expressed as means ± SEM. $^{***}p < 0.001$ and $^{**}p < 0.01$ are compared to the saline group; $\#\#p < 0.01$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group; $\dagger p < 0.05$ is compared to ATRA+LPS. The immunohistochemistry sections were prepared after the euthanization of animals following behavioral analysis. The saline, LPS, CAR+LPS, and FLX + LPS groups were taken as the first cohort ($n = 5$/group), while the ATRA+LPS, CAR+ATRA+LPS, and ATRA+LPS + FLX groups were from the second cohort ($n = 5$/group). LPS: lipopolysaccharide; ATRA: all-trans retinoic acid; FLX: fluoxetine; p-JNK: Jun N-terminal kinase; Bcl-2: B cell lymphoma-2.

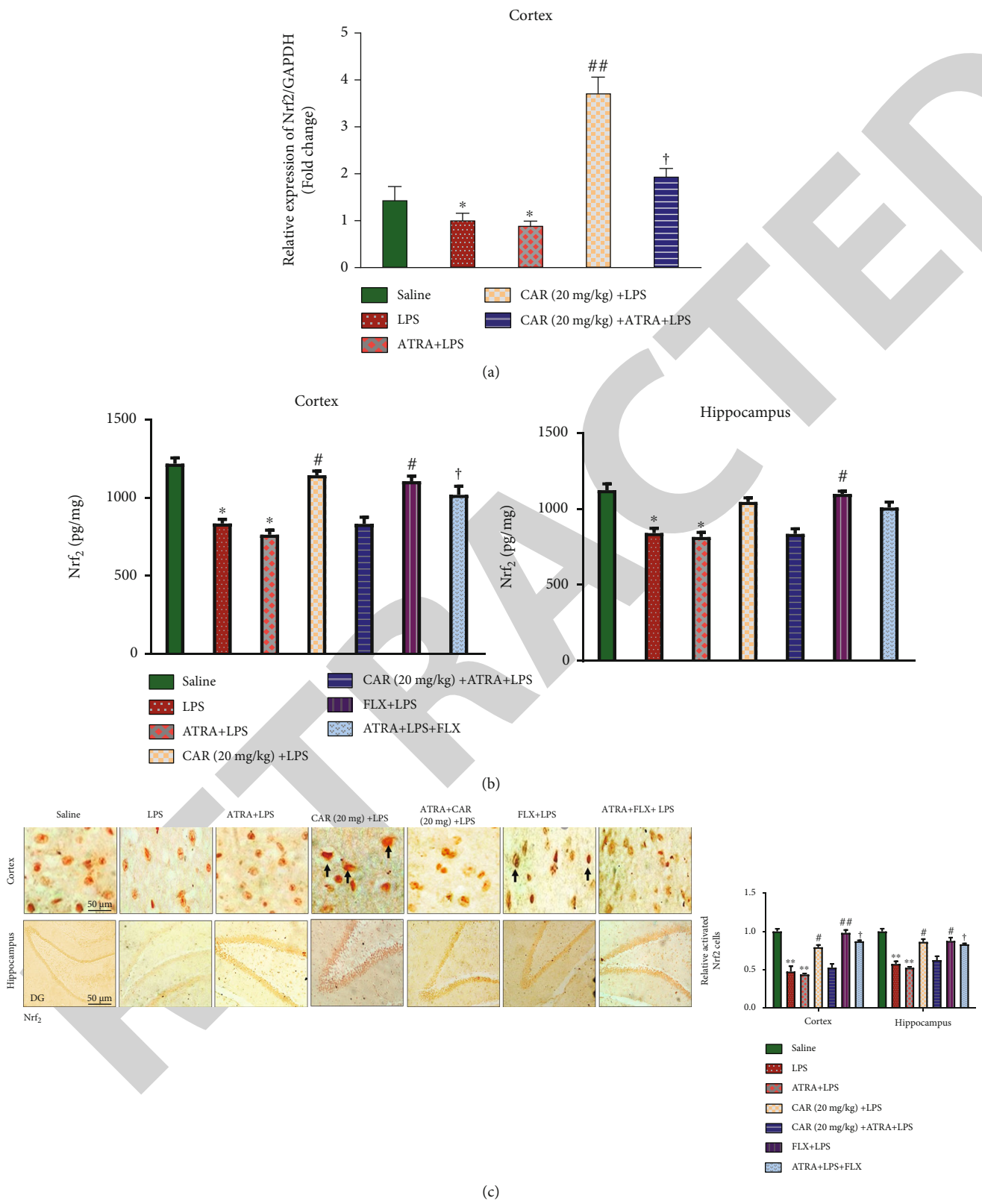
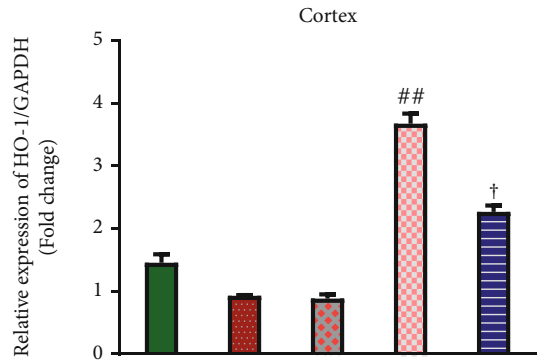
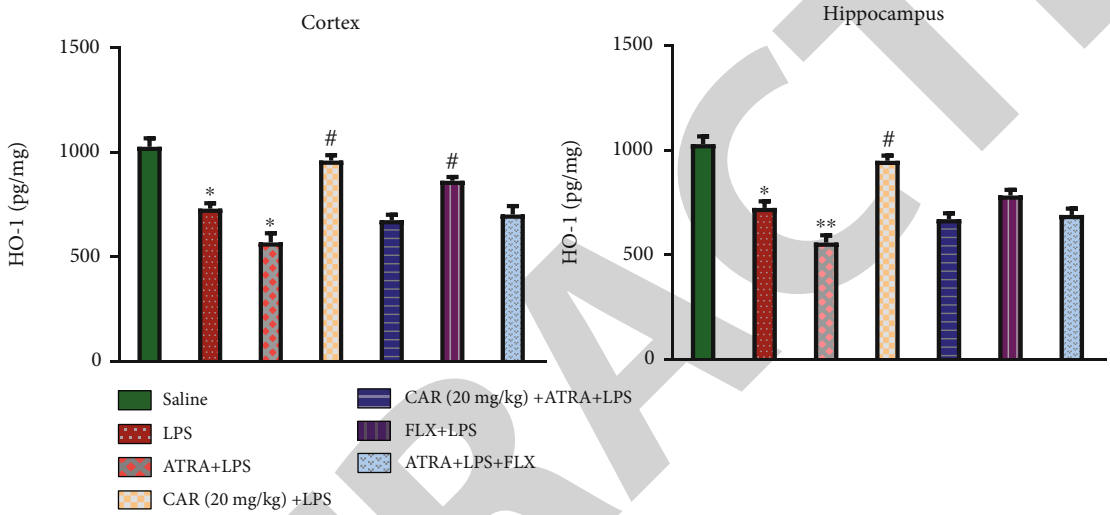


Figure 5: Continued.



(d)



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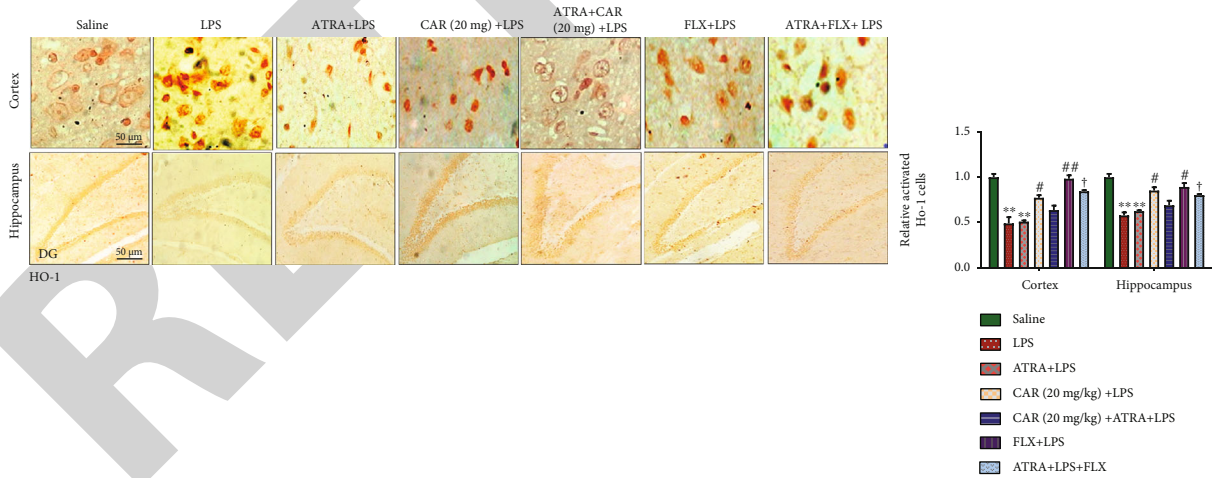

(f)

Figure 5: Effects of carveol on LPS-induced downregulation of *Nrf2* and *HO-1*. (a) RT-PCR analysis of *Nrf2* in the cortex. (b) Nrf2 protein level was measured by ELISA in the cortex and hippocampus. (c) Immunohistochemistry analysis of Nrf2 in cortex and hippocampus. Data are expressed as means ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$ are compared to the saline group; $\#\#p < 0.01$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group, while $^{\dagger}p < 0.05$ is compared to ATRA+LPS. A thick black arrow indicates nuclear localization of Nrf2 (d) RT-PCR analysis of *HO-1* in the cortex. (e) HO-1 protein level was measured by ELISA in the cortex and hippocampus. (f) Immunohistochemistry analysis of HO-1 in cortex and hippocampus. Data are expressed as means ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$ are compared to the saline group; $\#\#p < 0.01$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group, while $\dagger p < 0.05$ is compared to ATRA+LPS. The saline, LPS, CAR+LPS, and FLX + LPS groups were those studied in the first cohort ($n = 5$/group), while the ATRA+LPS, CAR+ATRA+LPS, and ATRA+LPS+ FLX groups were from the second cohort ($n = 5$/group). CAR 20: carveol (20 mg/kg); LPS: lipopolysaccharide; ATRA: all-trans retinoic acid; FLX: fluoxetine; *Nrf2*: nuclear factor E2-related factor; *HO-1*: heme oxygenase 1.

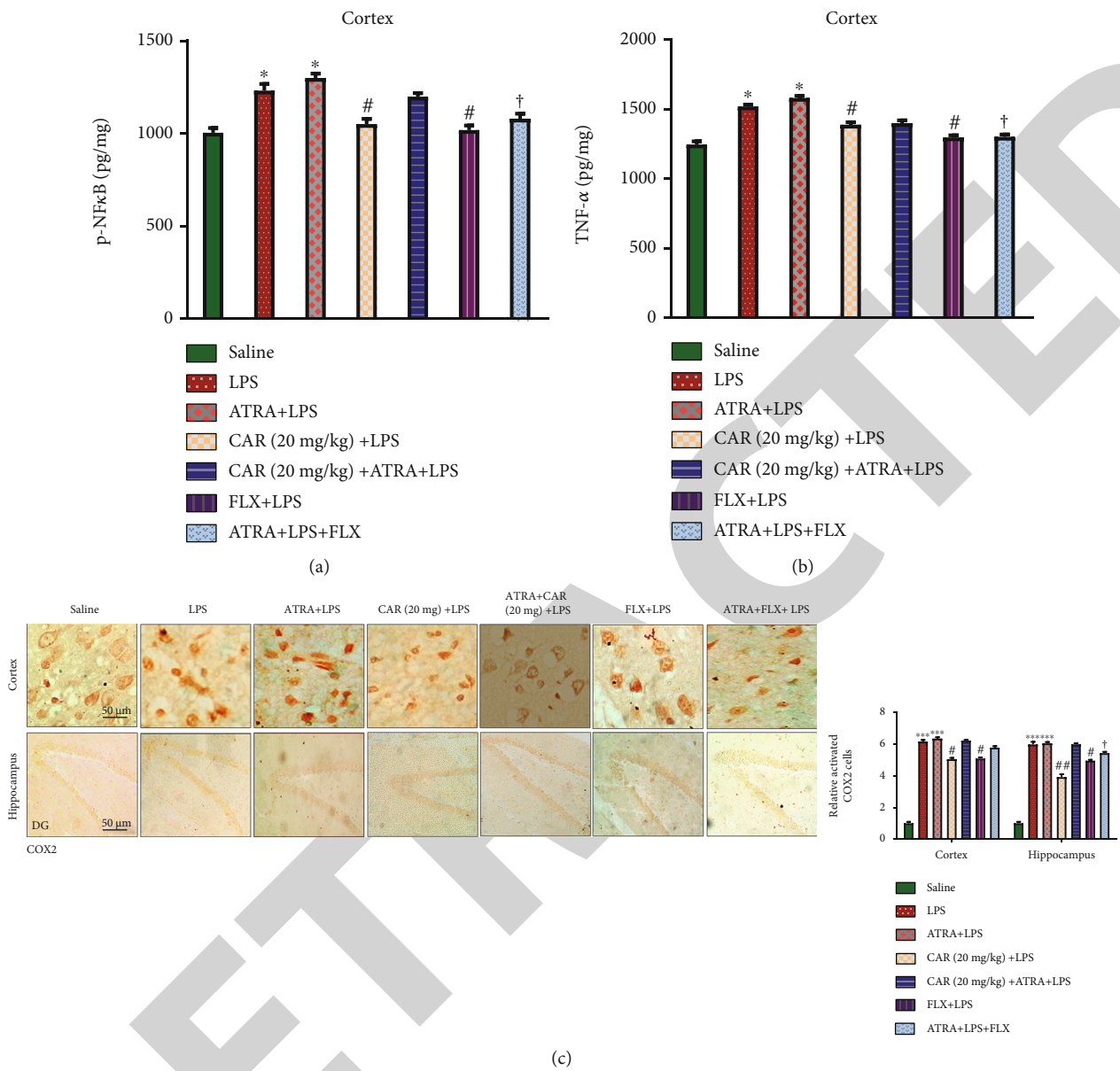

Figure 6: Effects of carveol on LPS-induced neuroinflammation. (a) p-NFKB and (b) TNF-α levels were measured by ELISA. Data are expressed as means ± SEM. (c) Immunohistochemistry results for COX-2 in the cortex and hippocampus. Scale bar 50 μm, magnification 40x. Data are expressed as means ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ are compared to the saline group; $\#\#p < 0.001$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group, and $\dagger p < 0.05$ is compared to ATRA+LPS. The saline, LPS, CAR+LPS, and FLX + LPS groups were those studied in the first cohort ($n = 5$/group), while the ATRA+LPS, CAR+ATRA+LPS, and ATRA+LPS+ FLX groups were from the second cohort ($n = 5$/group). CAR 20: carveol (20 mg/kg); LPS: lipopolysaccharide; ATRA: all-trans retinoic acid; FLX: fluoxetine; TNF-α: tumor necrosis factor-alpha; COX-2: cycloxygenase-2; p-NFkB: nuclear factor-kappa B.

4. Discussion

Research studies in the recent decade have highlighted the role of phytochemicals in maintaining the brain's chemical balance and providing a potential source of neuroprotectants in several neurodegenerative diseases [58]. The goal of the present research study was to reveal the neurotherapeutic potential of the natural product, carveol against LPS-induced depression and anxiety in the rodent model. Carveol was used in this study based on its reported anti-inflammatory, antioxidant, anti-Alzheimer, and other neuroprotective properties [46, 59].

Accumulating evidence has shown that peripheral immune activation and increased cytokine production contribute to the development of depression [60]. LPS administration in rodents induces depressive and anxiety-like behaviors in several ways that include reduced locomotor activity and a decrease in struggling time in an unfavorable environment such as FST [61]. Here, in this study, we have shown that carveol pretreatment significantly attenuated the LPS-

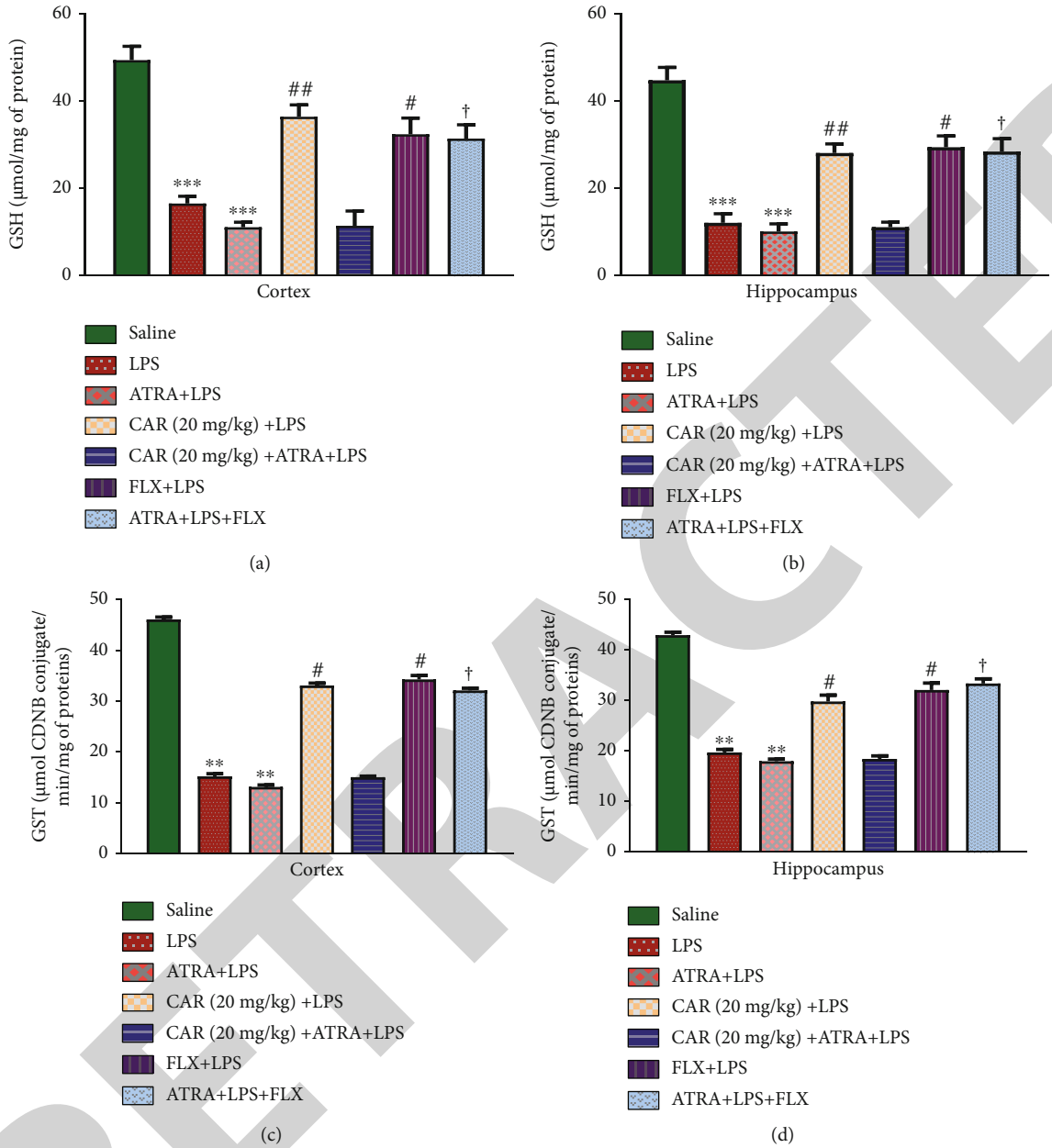

Figure 7: Continued.

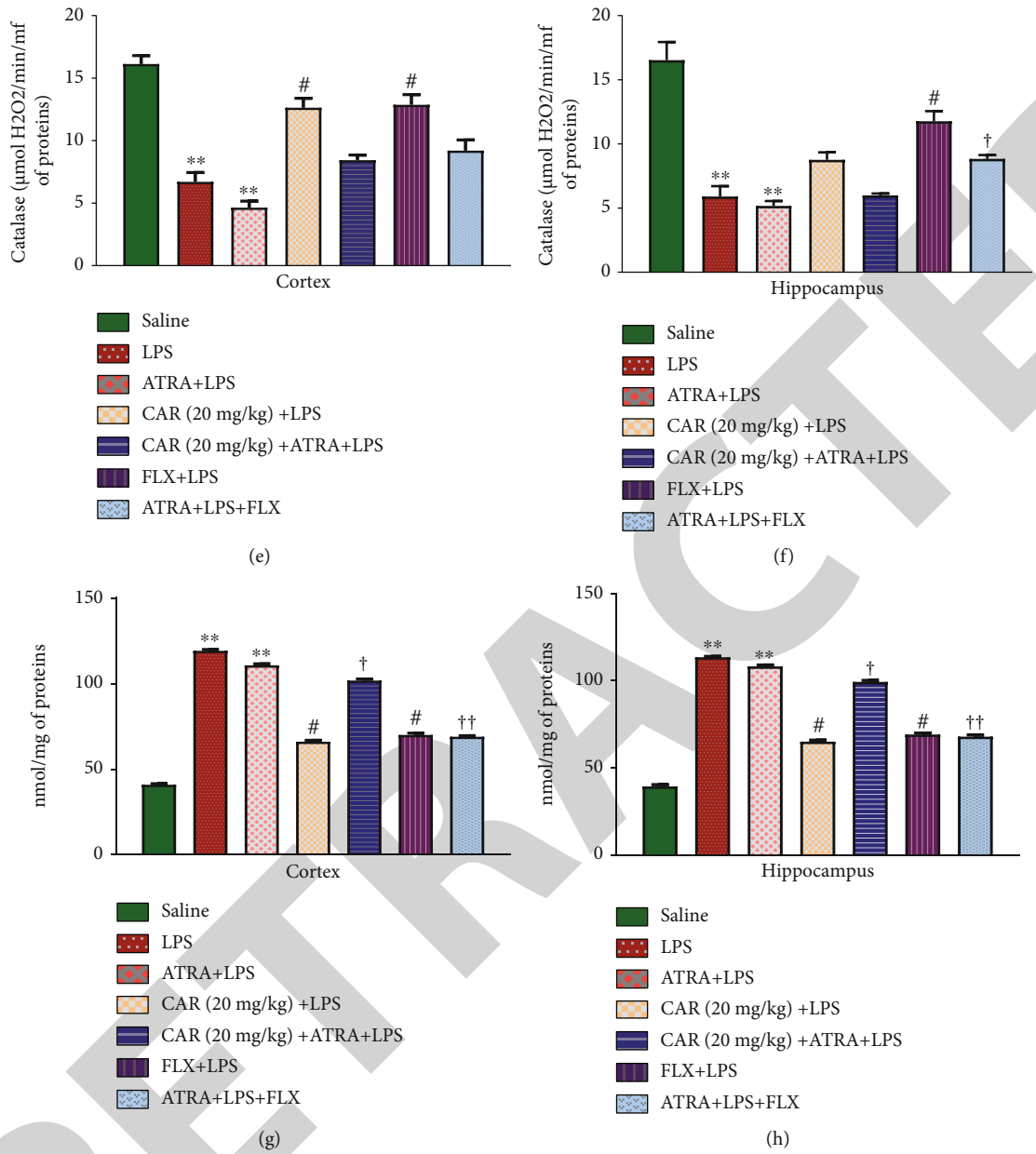

Figure 7: Effects of carveol pretreatment on antioxidant enzymes and LPS-induced lipid peroxidation. Effects of LPS and CAR on levels of GSH (a, b), GST (c, d), catalase (e, f), and TBARS (g, h). Data are expressed as means ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ are compared to the saline group; $\#\#p < 0.001$ and $\#p < 0.05$ indicate a significant difference compared to the LPS group, and $\dagger p < 0.05$ or $\dagger\dagger p < 0.01$ is compared to ATRA+LPS. The saline, LPS, CAR+LPS, and FLX + LPS groups were those studied in the first cohort ($n = 5$/group), while the ATRA+LPS, CAR+ATRA+LPS, and ATRA+LPS+ FLX groups were from the second cohort ($n = 5$/group). CAR 20: carveol (20 mg/kg); LPS: lipopolysaccharide; ATRA: all-trans retinoic acid; FLX: fluoxetine; CAT: catalase; GSH: reduced glutathione; GST: glutathione S-transferase; TBARS: thiobarbituric acid reactive substances.

induced behavioral despair by increasing mobility and struggling time. Previous research studies have reported that decreased locomotor activity in the FST may be caused by the increased level of proinflammatory cytokines and reactive oxygen species (ROS) in the LPS model [61]. In accordance with previous studies, our results showed that carveol (20 mg/kg) mitigated depression-like symptoms induced by LPS. In addition to FST, we determined the grooming behavior of experimental rats in the SST, which may indicate decreased motivation and apathy. Our results pointed out that LPS administration significantly decreases the grooming time, hence inducing apathy and lack of motivation. Administration of carveol improved the core symptoms of depressive-like behaviors by mitigating LPS-induced behavioral despair and apathy. Furthermore, we also investigated anxiety-like behaviors by EPM and LDB tests. The data obtained from these

paradigms determined the anxiolytic potential of carveol, as elevated values were observed in the open arm and lightbox parameters that indicate potential anxiolytic activity [62].

Several research bodies have demonstrated the involvement of oxidative stress and ROS in the pathophysiology of various neurological disorders [63, 64] including depression [65]. Our results attest to the previously reported data, that LPS administration induces an imbalance between endogenous antioxidant substances and stress agents, thereby stimulating the production of ROS and activation of the neuroinflammatory cascade. Furthermore, currently, several clinically used antidepressant drugs have potential ROS ameliorating effects in depressed patients. Among the various neuroinflammatory mechanisms, the *Nrf2* gene has been demonstrated as one of the major regulators of the cellular antioxidant system which involves transcription factor *Nrf2* and its downstream signalling protein HO-1 which modulates several anti-inflammatory and antioxidants genes [66]. Modulation of the Nrf2 pathway has been reported to play a major role in developing new strategies for neuronal protection [67]. Furthermore, the antidepressive activity of many protective agents has shown the upregulation of Nrf2 protein and its downstream signalling HO-1 in depression models [68]. Interestingly, our results are in line with previous studies indicating that the carveol-treated group reversed the LPS-induced increased levels of ROS and LPO and induces Nrf2/HO-1. These results validated our hypothesis that carveol may contain antioxidant potential and free-radical scavenging activity. To determine the possible mechanism for carveol-mediated neuroprotection, carveol along with ATRA (Nrf2 inhibitor) was administered to the LPS-intoxicated rats. Our results showed that inhibition of Nrf2 and HO-1 signalling pathways by ATRA treatment reversed the potential neuroprotective effects of carveol. Similarly, the biochemical assays also showed a significant decrease in levels of antioxidant enzymes (GSH, GST, and catalase) along with an increase in LPO and ROS levels in the ATRA-treated groups. This implies that Nrf2/HO-1 signalling pathway is a potential target in the antidepressant and anxiolytic activity of carveol. In accordance, our previous studies demonstrated that Nrf2 inhibition by ATRA increased infarction area in the MCAO model of ischemic stroke in rats [33]. Martín-de-Saavedra et al. stated that Nrf2 affects the monoamine mechanism of depression by modulating the level of different neurotransmitters such as dopamine, noradrenaline, and serotonin [36]. Furthermore, *Nrf2* knockout mice showed an increased level of glutamate accompanied by a significant decrease in the level of these neurotransmitters [36]. In addition, behavioral analysis in other research studies has shown an increase in immobility time in FST and a decrease in grooming time in SST in *Nrf2* null mice which further supports the role of Nrf-2 antagonism in depression [69]. It is well known that Nrf2 activation leads to the inhibition of inflammatory mediators via downregulating the NF-κB signaling pathway. HO-1, downstream of Nrf2 is also known to be a potent inhibitor of proinflammatory cytokines [70, 71]. Consistent with previous research studies, our findings demonstrated that carveol effectively reversed the LPS-induced protein expression of various inflammatory mediators such as p-NFkB, TNF-α, and COX-2. Similarly, carveol upregulated the LPS-induced decreased protein expression of the antioxidant enzymes HO-1. Mechanistically, both the anti-inflammatory and antioxidant activities of carveol were diminished in the ATRA-treated groups, which supports our hypothesis that carveol exerts its protective activity via activating the Nrf2/HO-1 signaling pathway [71, 72].

Several lines of evidence have reported the involvement of MAP kinases such as JNK signaling in the induction of neuroinflammation-induced neurodegenerative disorders. Activation of the JNK also interferes with the BCL-2 family of proteins to induce cell death via activation of the mitochondrial apoptotic pathway [73]. In accordance, other studies have demonstrated a close interplay of JNK and caspase-3 in the apoptotic cascade [74, 75]. Our results demonstrated that carveol alleviated LPS-induced elevated levels of proapoptotic proteins and p-JNK.

5. Conclusion

Taken together, we can conclude from our results that carveol possesses potential antioxidant, anti-inflammatory, and neuroprotective properties. Further, our proposed neuroprotective mechanism suggests that carveol activated the endogenous antioxidant proteins like Nrf2 and HO-1 coupled to the downregulation of anti-inflammatory proteins like p-NFκB and p-JNK. Collectively, these protective properties of carveol may offer a new therapeutic option for protecting the brain from neuroinflammation and oxidative stress.

Data Availability

The research data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

Acknowledgments

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